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ECOLOGICAL AND GEOGRAPHICAL VARIABILITY OF SOIL BACTERIA

Ye.N.Mishustin

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B.L.Isachenko, Editor

Basic aspects of soil microbiology from the viewpoint of geographical ecology are reviewed, with emphasis on various subspecies of B.mycoides isolated from Russian soils of differing type (chernozem, podzol, gray soil, steppe soil, etc.) and differing geographical location. B.mycoides preferably uses the nitrogen of organic compounds, with a few subspecies able to grow on ammonium salts. Most cultures yield sinistral colonies, with the direction of rotation being a hereditary trait, only temporarily influencible by change in nutrient composition. Geographical races differ in behavior to salt concentration of media, with the southern races, because of higher intracellular pressure, being more halotolerant. Darwin's laws for higher organisms are extended to microorganisms by proving the existence of ecological types of bacteria, regularly succeeding each other on progressing from North to South. Use of typical bacteria as indicator organisms for soil-forming processes in various climates and for determining the origin of grain and other seed material is discussed. Extensive tabular material on optimum and maximum temperature for various bacteria species, nutrient media, fermentative power for sugars, cell widths, spore size, thermal death point, etc. and photomicrographs of growth stages are included.

Author

EDITOR'S PREFACE

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The monograph by Dr. Ye.N.Mishustin, Doctor of Biological Sciences, "The Ecogeographic Variability of Soil Bacteria", which reports and summarizes many years of research, is of undoubted interest for theory and practice. The author has succeeded in showing that certain microorganisms which were considered cosmopolites, such as B.mycoides, have ecological races that differ sharply in cultural, morphological, and physiological properties.

The patterns noted for B.mycoides are apparently common to the world of microbes, but the qualitative expression of the changes or shifts under environmental effects vary greatly from species to species of microorganisms. Future work will more closely define these features, but will by no means diminish the importance of this book which, for the first time, gives extensive treatment to the question of the influence of climatic and ecologic conditions on the proper-

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* Numbers in the margin indicate pagination in the original foreign text.

ties of bacteria.

The material accumulated by Ye.N.Mishustin makes it possible to apply the laws established in studying the geographical variability of higher organisms to the world of microorganisms. This again confirms the ingenious concept by Darwin who was the first to raise the question of the adaptive variability of animals and plants under the influence of climate.

The practical value of Mishustin's work is obvious. It should capture the interest of microbiologists in various fields of technology and agriculture. More specifically, in the utilization of microorganisms for antibiotics, their geographic variability should be taken into account, an aspect that opens up broad and important prospects.

The possibility of making use of the phenomenon of geographic variability for various practical purposes is illustrated in this book on a number of typical examples.

Academician B.L.Isachenko

PREFACE /5

One of my studies on the question of the adaptation of soil bacteria to the temperature conditions of a given climate was published twenty years ago in the Journal "Pochvovedeniye" (Agronomy). I demonstrated there that the cardinal temperature points vary sharply for saprophytic bacteria, depending on the conditions of the environment in which they live. This led to the postulate that bacteria, like higher organisms, comprise ecological races differing in a number of traits. This view, which essentially is an application of Darwin's concept to the world of microbes, was fundamentally new since scientists who advocate the cosmopolitanism of bacteria were convinced that the environment had no appreciable influence on their characteristics.

In verifying his hypothesis of the existence of ecological races among bacteria, I became specifically concerned with an analysis of the variability of the soil bacterium B. mycoides which had been thoroughly studied and which, because of its peculiar cultural properties, is easy to determine. My work showed that this bacterium comprises several well-defined geographical races, differing in cell size, colony structure, and physiological traits.

On a number of examples from this work, I demonstrated that variability under the influence of climate is encountered in various bacteria, meaning that it is of a universal character.

I am well aware that this problem cannot be solved by a single researcher, and I will be highly gratified to learn that others may become interested in the work I started here. The application of the doctrine of geographic variability of bacteria to an analysis of the formation of soil types seems of great promise. The conclusions of my work may also be useful in other disciplines of science.

In conclusion, I wish to mention that I was greatly assisted in this work by several colleagues, especially by V.A.Mirzoyeva who, for a number of years, /6 participated in elaborating several of the Chapters. In recent years, V.K. Mishustina was of great help in the experimental work.

I express my profound gratitude to Academician B.L.Isachenko, Director of the Institute of Microbiology, USSR Academy of Sciences, and to his assistant, A.A.Imshenetskiy, corresponding member, USSR Academy of Sciences, who cooperated in the publication of this work and gave valuable advice in its arrangement.

I am grateful, for a number of critical remarks, to the members of the Academic Council of the Institute of Microbiology, USSR Academy of Sciences, who went over the manuscript.

Author

CHAPTER I /7

ADAPTATION OF ORGANISMS TO ENVIRONMENTAL CONDITIONS

1. Geographic Variability in Higher Organisms

Up to the present, several geographic laws have been established as to the variation of the morphological and physiological properties of higher organisms. Thus, according to the law discovered by Bergmann, the body size in the same race of animals increases in the direction from South to North. Corresponding variations were found to exist in the structure of the limbs and appendages of the body (Allen's rule), including variations in coloration of the animals, etc. In plants, growing in different latitudes, the composition of the fats shows regular variations, and shifts in the protein ratios are noted.

There can be no doubt of the adaptive character of such variations. Some of these differences resulting from the existence of organisms in different environments are not inherited, i.e., they constitute modifications. However, a significant number of traits that appear as a result of the protracted action of climatic and biological factors are hereditarily fixed, which suggests the existence of geographical races characterized by several morphological and physiological traits. "The term 'race' ",writes V.L.Komarov, "is used by us to denote individual groups which, while differing among themselves in relatively unimportant traits, nevertheless transmit these traits from generation to generation."

All traits of the organism, from the most insignificant to the most important, are subject to racial variation. There are differences, not only of morphology but even of physiology, between closely related organisms.

The geographic races differ among themselves as a rule in a number of traits. This is only natural, since the environment acts in many ways on the organism.

Darwin noted that natural selection affects the entire organization of the organism, since the appearance of some mutation necessarily leads to the appearance of others. According to the formulation of his law of associated variability, the individual fortuitous variations of certain parts of the organism lead to a simultaneous appearance of other new characteristics. Some of the new traits may be regarded as fundamental, according to which selection operates, while others may be considered merely subordinate. /8

In his works on the variability of living species, Darwin repeatedly mentions the regular variation of organisms in response to geographical factors. Thus, his work "The Variation of Animals and Plants under the Influence of Domestication" contains a chapter on "Direct and determinate action of the external conditions of existence", discussing the origin of physiographic ecology of animals and plants. In his book "The Origin of Species", Darwin

repeatedly mentioned the geographic species or subspecies of Columbia livia, which show only slight mutual differences. He described the existence of geographical forms and closely related species that follow each other in sequence, from North to South.

Darwin considered the development of geographic varieties as entirely dependent on natural selection, but not the result of a direct influence of the environment on the organism. He associated the phenomenon of geographic variations with ecological factors, i.e., as adaptive modifications. In this sense, he attributed particular importance, in the formation of types, to transmitted variations.

Darwin wrote: "...I consider selection to be the most active cause, both in the domestication of races by man and in the evolution of natural species".

The above proposition logically leads to the conclusion that the origin of geographic series can only be explained by the theory of natural selection, provided that the principal geographic variations are favorable to the organisms composing such series.

In his studies, Darwin did not consider in detail the motive forces responsible for the variation in the inherited traits of the organisms. At the same time, it follows from certain of his assertions that he viewed the manifestations of life as a struggle between traits fixed by heredity and those acquired during life (see, for example, Darwin's remark on the training of horses and dogs).

A similar opinion was expressed by Engels in his discussion of the improvement of the human hand. Engels writes: "The hand is thus not only an instrument of work but also its product. Because of this work, its adaptation to new operations, its transmission to posterity of the resultant modifications (specifically the evolution of muscles, tendons, and eventually of bones), and finally because of constant application and reapplication of such increasing perfection to more complex functions, has it ultimately become possible for the human hand to reach the extraordinary perfection at which, as by some magic power, it was able to accomplish such creative work as Raphael's paintings, Thorwaldsen's statues, and Paganini's music". /9

Engels placed exceptional emphasis on natural selection in the life struggle in an unstable environment (variations in geographic and climatic conditions); he stated specifically that this obsoletes any Malthusianism and that, even if this latter philosophy could be admitted here, the above process would not be changed or, at most, accelerated.

The above postulates were formulated by Soviet Darwinists (Michurin, Lysenko, and others) who believe that training plays the basic role in the modification of hereditary traits of the organism.

Thus, for the higher organisms, not only a number of laws for the variation of their properties under the influence of geography have been established, but also the theoretical principles for explaining the origin of such variability were developed.

This brings us to the great interest of similar work on bacteria, i.e., on unicellular organisms without structural cell system or sexual processes.

Unfortunately, until very recently there had been almost no such studies, either abroad or in the USSR. To a certain extent, this book is to fill an existing gap. It is devoted primarily to the study of the variations in morphological, cultural, and physiological traits of one of the typical soil bacteria, Bac.* mycoides, under the influence of ecogeographic conditions. As follows from the discussion in the entire book, our work has convinced us of the existence, among bacteria, of geographic races differing sharply in several basic traits. These traits are firmly fixed by heredity and are transmitted to the progeny.

The generality of the adaptive reactions of soil bacteria to the climate is shown on various typical examples.

Thus, the material that follows is to promote the application of the principles of Darwinism to the world of microbes.

In the introductory Chapter, we will restrict ourselves to extremely brief remarks on the fundamental topics of the geographic variability of living /10 organisms. There is an excellent presentation of this subject in Lukin's fundamental work "Darwinism and Geographical Patterns in the Variations of Organisms".

2. Variability of the Traits of Microorganisms under Environmental Influence

Miehe (1906) raised the question of the geography of microorganisms in a general form. He stated that this field was still terra incognita, "although the knowledge of the natural habitats of pathogenic microorganisms is of fundamental significance for the etiology and prophylaxis of infectious diseases". In fact, it is well known that the spread of various diseases is confined to certain definite regions.

Of course, this question is of the same practical and theoretical importance in the study of saprophytic microorganisms.

With respect to microorganisms, especially bacteria, many of which can be considered as cosmopolitan with a habitat extending from the extreme North to the tropics, variability under the influence of climate has never been systematically studied. However, even on the basis of purely theoretical considerations and individual and scattered indications, reliable data should have been possible on the variability of microorganisms under environmental influences. For example, it is well known that the species of lactic acid bacteria, encountered in nature, alternate at various latitudes.

The work by Vinogradskiy (1892) suggests the existence, in soils of different climatic zones, of nitrogen-fixing bacteria that are far from identical.

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* Hereafter, abbreviated as B.mycoides.

The Javanese Nitrosomonas differs sharply from the European form in its small size and unusually long flagellum. The European Nitrobacter, according to Vinogradskiy, grows in an entirely different manner on liquid nutrient media than the Javanese form of the same bacteria.

In addition to the above examples, there are facts pointing to the absence of certain bacteria from certain climatic zones, or to extensive variations in the properties of so-called cosmopolitans under the influence of the climatic factor.

Thus, Löhnis and Pillay considered that the nitrobacter in Indian soils is replaced by a different nitrogen-fixing microorganism, B.malabarensis.

De Kruyff (1910) asserted that the distribution of thermophilic bacteria is limited to certain geographic areas. He considered them to be bacteria of the waters and soils of tropical countries.

This view, however, has been disproved by our own studies. /11

Isachenko (1914) noted the absence of rose yeasts from the Arctic, North of 54°N.

The literature contains reports on unsuccessful attempts to isolate bacteria of the Proteus type in the Antarctic.

Prior to the publication of our investigations on the temperature adaptation of soil bacteria to given climatic conditions, Kazanskiy (1932) established that many bacteria of northern soils grow better at lower temperatures.

Isachenko (1934) who studied the soil in a number of Arctic islands, shares this opinion. He also noted a lowering in the growth temperature in bacteria of northern localities in earlier work (1914).

Butkevich (1935), in investigation on marine bacteria of the northern latitudes, found that some types, such as Spirophyllum ferrugineum had a lower optimum growth point, sometimes below 10°C.

Of course, associations of psychrophilic and thermophilic microbes may have occurred among the usual forms by a prolonged process of adaptation. A certain proof is constituted by observations of Yelenkin, who noted that the thermophilic algae of the hot springs of Kamchatka are actually forms of cold-water algae adapted to high temperatures. Many species of these algae formed stable races which, under the conditions of existence in nature, are no longer found in water at the usual temperature. It is true that Arnoldi states that some thermophilic algae (for instance, Hapalosiphon laminosum) might still be considered relics, surviving from geological times when the temperature of all water on the earth was considerably higher.

In analyzing the relationship of microorganisms with the temperature, Lyubimenko wrote: "By adaptation to a definite optimum temperature, the organism strives to reflect the predominant temperature of its environment. For this reason, for instance, pathogenic bacteria that developed at the temperature of

the human body show an optimum at 37° C, while the optimum for most saprophytic bacteria lies between 20° and 35° C, corresponding to wider fluctuations of the ambient temperature."

The adaptation of the organism to environmental temperature conditions most often involves corresponding shifts of the necessary minimum and maximum. However, cases have also been noted when the adaptation primarily affected the position of the maximum. For instance, many thermophilic algae among the /12 diatomaceous desmidia and blue-green algae, are able to grow in water at a temperature of 50 - 90° C but are also encountered in waters at lower temperatures.

The osmotic properties of the microbial cell also reflect the features of the environment. Thus, marine bacteria are highly sensitive to the presence of a certain salt minimum. They grow best in concentrated nutrient media but not in diluted ones.

It is interesting to note that marine halophilic microorganisms react strongly, and occasionally even die, if the NaCl is replaced by other salts in equimolar concentrations, except for Na_2SO_4 and $NaNO_3$. Thus, NaCl is necessary as such for these bacteria.

At the same time, according to Dianova and Voroshilova, fresh-water bacteria grow well in both ordinary nutrient media and in media with a higher salt concentration.

These phenomena can be explained by assuming that all fresh-water bacteria and, to a still greater degree, terrestrial bacteria, live in environments subject to great fluctuations in salt concentration and salt composition. Marine halophiles, however, are aborigines of the sea, i.e., denizens of a medium whose composition is constant and the same in all of the global oceans (Shokal'skiy). Variations in the composition of ocean water relate to fluctuations of salt concentration but not of salt composition.

Thus, the conditions of microbal life place their stamp on the variability of their cells. Nevertheless, protracted action of a certain medium will result in a corresponding modification of bacteria with even the most persistent traits. For example, Rubenchik demonstrated the transformation of obligate halophilic bacteria of the Odessa marshes into facultative forms, when the water gradually freshened. Similar phenomenon were noted by Lyubimenko for the brown and red algae which, as a rule, are denizens of the sea. At the same time, some representatives of these algae are also known to live in fresh water. In all probability, they have gradually migrated from the river mouths into fresh water.

Certain studies in the field of interest, not mentioned here, will be discussed in later Sections of this monograph.

An excellent illustration of the generality of adaptive reactions to climatic conditions in widely differing microorganisms is given in the interesting paper by Smaragdova (1941) who showed that the optimum temperature of the /13 division rate in the paramecium is not the same for different geographic forms of these organisms. Protozoa were found to react like bacteria to the climatic conditions.

In certain forms (Paramecium caudatum), the optimum temperature of the division rate increases from North to South and is directly dependent on the average summer temperature of the air.

The body size of Paramecium bursaria varies from North to South. The largest were the northern forms and the smallest the southern.

The distinctive traits of the geographic forms of protozoa are hereditary. The relatively few data, available on the geographic variability of bacteria, are nevertheless sufficient to apply the laws discovered for higher organisms to the world of small organisms.

Extensive laboratory data fully confirm the possibility of variation in bacteria traits under the influence of the environment. We will present several examples of adaptation to temperature and to osmotic pressure, i.e., reactions that are especially manifest for ecogeographic variability. For example, as far back as Dallinger (1887), successful trials were made to adapt protozoa for growth at rather high temperatures by prolonged acclimation. He obtained a thermostable race by gradually raising the temperature. Such experimental variants of the protozoa were able to grow at temperatures lethal to the original culture.

Somewhat later, Dieudonné (1894) by using suitable cultural conditions was able to develop psychrophilic and thermophilic forms of the anthrax bacillus. He also obtained, by means of adaptation, forms of B.fluorescens and B.prodigiosum with a higher temperature maximum of growth.

Similar experiments with B.subtilis and certain other microbes were staged by Tsiklinskaya (1899) who, among other things, noted that not all bacteria could be adapted with the same ease to new temperature conditions.

Löwenstein (1903) adapted the thermophilic alga Hapalosiphon laminosus which has its maximum at 52° C, to lower temperatures. Prolonged culture of this alga at room temperature so modified its requirements that it lost its ability to grow in hot water.

Imshenetskiy and Loginova (1944) obtained yeasts with entirely satisfactory multiplication at superoptimal temperatures. /14

Of course, acclimatization, which is successful under laboratory conditions, is also widespread in the natural habitat.

These facts, and a number of similar ones, suggest that the environment has a major influence on the traits of microorganisms.

Having the facilities for isolating bacteria from soils of various climatic zones, we placed special emphasis on analyzing their possible relation to the temperature and osmotic properties of the medium, and also attempted to establish a correlation between the climatic indices and the corresponding traits of the microbes. These data were selected by us as the basic elements characterizing the climate. We also paid considerable attention to studying the effect of climate on the variation of cultural and physiological traits of the bacteria of

interest here.

B.mycoides was selected for the basic studies. Selection of this particular species was prompted by various considerations. First, B.mycoides, considered as one of the most effective soil ammonifiers, is widely distributed in various types of soil. Second, its colonies are of so specific a character that its identification is not particularly difficult. For work in this direction, the above property was most important.

It must be noted that the problem of geographic races of soil microorganisms is not only of theoretical interest but also of great practical importance. For example, only a very limited number of strains of rhizobia and nitrobacter are being used at present for the widely practiced bacterization of soil, without considering the features of soil type or climatic zone. Nevertheless, it must be assumed that nitrogen-fixing bacteria, living in different climatic zones must have some distinguishing features; already today, we have some indications of this.

A proper study of the ecological races of nitrogen-fixing bacteria will bring up the question as to their utilization in agriculture, on a fully scientific basis.

No less interesting results can be expected from an ecological study of microorganisms to be used in the dairy industry, brewing, antibiotics production, and other branches of the national economy.

3. Geographical Ecology of Soil Microorganisms /15

In working out the problems connected with defining the existence of geographical races among the soil microflora, we never lost sight of the problem of the geographical ecology of soil bacteria. The general difficulties in the latter field are responsible for the slow progress in the accumulation of the necessary data. Nevertheless, the material assembled so far is rather extensive and the author,who has done much work on soils of various types, hopes to publish it in a special review.

Here, we will merely mention some data relative to the distribution of lower organisms over certain areas. The main controlling factors in the distribution of microorganisms are: a) physicochemical features of the soil, and b) humidity and temperature, i.e., the climatic conditions.

Several facts suggesting the existence of natural regions of distribution of fungal forms are known today; these regions are determined by climatic conditions.

Systematizations of facts of this nature all refer to the more thoroughly studied higher fungi and phytoparasites. The eminent mycologist Yachevskiy considered geographical mycology or mycogeography, however, to be merely a science in its very inception.

Yet it is obvious that certain lower fungi, constituting the soil flora,

are far from being cosmopolitan. For example, in studying the southern soils (gray soils or serozems) we frequently encountered fungi of the species Choanephora, which are absent from northern soils. It is well known that the most northerly species, Penicillium glaucum, is replaced in the Mediterranean region by other species: Penicillium italicum, Penicillium digitatum, etc.

According to Isachenko, Rhodotorulae are encountered only within certain climatic zones.

No verified data on the distribution of certain species of bacteria within certain climatic zones are available. However, one can speak of geographical ecology of soil bacteria to the extent to which the soil background itself affects the distribution of bacteria in a definite manner. In this respect we could mention the nitrobacter whose presence in soil is linked to several conditions (supply of potassium and phosphorus, moisture, and reaction of the medium).

In an original work, Jamogato and Itano showed that different species of nitrobacter have differing optimum pH. For this reason, depending on the acidity of the soil, one or the other species of nitrobacter will predominate. /16

In our work, we demonstrated a cellulosolytic microflora that had an entirely different composition in soils of different cultivation. For example, virgin podzols and chernozems contain very many fungi and mycobacteria but, as a rule, no Cytophaga. The latter bacterium increases in number in more cultivated soils and becomes the dominant form in intensely fertilized soils. It is also noted that some species of mycobacteria occur in podzolic soils, and other species in chernozems.

These observations were later confirmed by Sorokina.

Vinogradskiy, in speaking of nitrosobacteria, states that soils of different fertility contain different species of these microorganisms.

We obtained analogous results for urobacteria.

The composition of sporulating bacteria is far from being the same in soils of different types.

All these summarily listed facts, even at this early date, indicate not only the necessity of a more detailed study of the geographical ecology of soil bacteria, but also of a practical utilization of bacteria as indices of soil condition.

CHAPTER II /17

NATURAL AND EXPERIMENTAL VARIANTS OF B.MYCOIDES

1. Synonyms

B.mycoides, which forms highly characteristic colonies on the surface of solid nutrient media (Fig.1), was first described by Flügge (1886). This bacterium has been rather well studied by now, especially the macrostructure of its colonies. This has recently led to studies on the phenomena of dissociation and variability of bacteria.

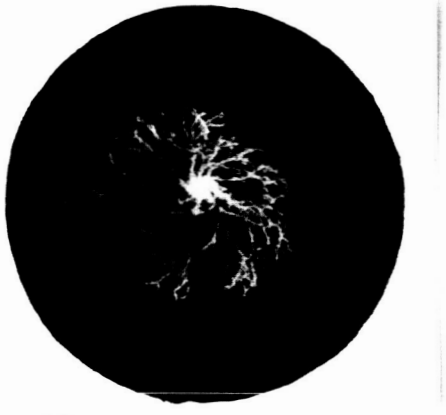


Fig.1 Colony of B.Mycoides on Peptone Agar

The work in this direction permitted defining the existence of several subspecies (variants) among B.mycoides, whose distribution, as shown in the present work, is connected with certain soil zones. This fact, in particular, made it possible to speak of the existence of geographic variability in bacteria.

Whether a given culture of B.mycoides belongs to one or another variant can be determined by an analysis of the macrostructure of its colonies. Therefore, we will first give a description of the formation of a colony of B.mycoides and of the main structural differences in the various subspecies. In this work we used both our own material and data from the literature.

B.mycoides has been studied under different names by a number of scientists. In the opinion of Gottheil (1901) and others, the following bacteria may be /18 given as possible synonyms for B.mycoides:

Wurzelbacillus Eisenberg (1886.
Bacillus figurans Crookshank (1886).
Bacillus ramosus Eisenberg (1891).
Bacillus pseudoanthracis Wahrlich (1891).

Bacillus ramosus Frankland (1889).
Wurzelbacillus Fränkel (1890).
Bacillus radicosus Zimmerman (1891).
Bacillus implexis Zimmerman (1891).
Bacterium casei Adametz (1889).
Bacillus intricatus Russell (1892).
Bacillus brassicae Pommer (1886).

In Gordon's opinion, B.mycoides may perhaps be the same as B.fluorescens Flügge, B.albolactis Migula, B.praussnitzii Trevisan (B.ramosus liquefaciens Flügge).

Rautenshteyn claims that B.undulatus den Dooren de Jong is also identical with B.mycoides.

It might well be that the species of bacteria described by Holzmüller under the names B.effusus, B.olfactorius, B.nanus, and B.dendroides are likewise subspecies of B.mycoides.

Krasil'nikov (1947) asserts that the following bacteria are very close to B.mycoides:

Bacillus subfiliforme Henrici (1894), syn. Bact. subfiliforme Migula (1900).
Bacillus fulminans Schrine et Grenfield (1930).
Bacillus adherans Lanbach (1916).
Bacillus articulatus Kern (1897).
Bacterium tenax Kern (1896).
Bacillus cereus Frankland (1887).
Bacillus ellenbachensis alfa Stutzer (1898).
Bacillus ellenbachensis Gottheil (1901).
Bacillus vermicularis Frankland (1889).
Bacillus stolonifer Phol (1892).
Bacillus albolactis Migula (1900).

It should be noted that a number of forms described as B.mycoides have no relationship to this microorganism. For example, the B.mycoides corallinus Hefferan, isolated from the water of the Mississippi (1900) is assigned by Bergey to the genus Serratia, under the name of Serratia corallina. This bacterium is assigned by Krasil'nikov to the genus Myxobacterium.

The same holds true for B.mycoides roseus Scholl (1889), which is called Serratia rosea in Bergey's Manual of Determinative Bacteriology.

As evidenced by several basic properties, B.mycoides bruneus whose traits /19 were given by Perlberger cannot be classified with the species of interest to us.

2. Formation of a B.Mycoides Colony

The structural characteristics of B.mycoides colonies, like those of other

microorganisms, have been studied by most authors on meat-peptone agar (MPA). This tradition has been followed in the present work; for this reason, in describing the growth dynamics of B.mycoides colony, we will concentrate largely on colonies grown on MPA.

As pointed out above, B.mycoides forms characteristic colonies on MPA, which appear to consist of tightly intertwined strands with a tendency to become circinate, usually in a counterclockwise sense (sinistral). These strands consist of several rows of parallel bacterial filaments.

On the basis of work by Marshall Word (1895), Wahrlich (1901), Holzmüller (1909), and Pringsheim and Langer (1924), the growth of a B.mycoides colony can be described as follows: The germinating spore puts out a rod, which begins to grow rapidly. According to data by Marshall Word, the growth of the bacterial filament formed by B.mycoides, under favorable conditions, may be 0.75 μ per minute (45 μ per hour). According to Holzmüller, a filament of B.mycoides can lengthen to 180 - 240 μ within one hour. According to Gottheil, a B.mycoides filament may be as long as 10 mm after 12 - 20 hours.

Septa are noted in bacterial cells grown to several tens of microns.

Pringsheim and Langer stated that, after germination of the spore, its position in the medium will not change. This makes one assume that the spore adheres to the surface of the nutrient medium, since otherwise it would be displaced from its position by the pressure of the growing bacterial filament. When several spores were placed on the nutrient medium, their mutual position after germination did not change, by virtue of this cause.

In general, the development of the bacterial filament can be clearly observed under the microscope if a preparation in a wet chamber is used. Here, the original formation of the bacterial filament and its subsequent division are easily established.

All investigators who studied the growth of B.mycoides emphasize that the growth of its pseudomycelium is intercalary. It is of course difficult to say whether the growth of each developing cell of the filament proceeds from one /20
end only, or whether the cell lengthens uniformly. Pringsheim and Langer attempted to determine this by sprinkling a filament of B.mycoides with finely divided chalk, so as to mark the growth of the cells. The bits of chalk, however, did not adhere to the bacterial filament.

A young bacterial filament appears to have a homogeneous structure under the microscope. It is surrounded by a film of capillary retained water.

When the filament reaches a length of 30 - 40 microns, and has already divided, it usually begins to bend. On further growth, this bending increases. Most cultures of B.mycoides give filaments with predominantly a counterclockwise curvature (sinistral forms). Diametrically opposed forms (dextral forms) are also encountered. After germination of the spores, the direction of the primary filaments is purely random. However, their tendency to either a sinistral or dextral rotation, is soon determined, thus also fixing the general orientation of the colony.

In the growing filament at the place of arcuation, cell separation often occurs (Figs.2, 3). The ends of the cells freed at the site of the break continue to grow, forming side branches. Such breaks may repeat many times during the growth of a colony.

Fig.2 Formation of a Colony of B.Mycoides (according to Holzmüller)

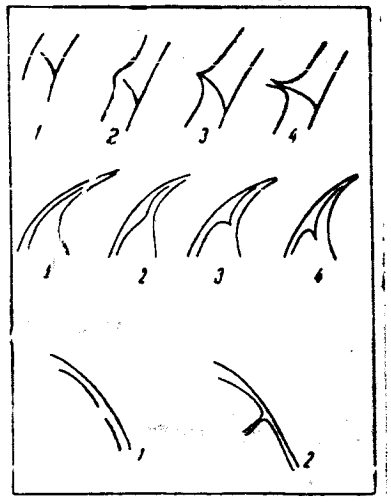


Fig.3 Formation of Branches in the Bacterial Filaments of a Colony of B.Mycoides (according to Holzmüller)

Occasionally, the individual cells forming a filament, at the point of their contact, start moving sideways and give rise to a new filament. The frequency of such bifurcations determines the more or less dense overgrowth of the agar /21 sector on which the B.mycoides colony is growing.

When the filament grows in length, some sort of resistance may cause it to arc or even to form a loop. Such a loop may break, and each end of it then resumes its growth.

Breaks of this kind may be repeated, so that instead of a single primary filament a number of filaments will appear on the medium, proceeding either parallel to each other or at a certain angle.

If the growing filament encounters a mechanical obstacle, it begins to bend, and its end creeps along the body constituting the obstacle. This picture is also observed when two filaments meet.

Crossing of individual filaments is a much less frequent occurrence and is possible only at right angles. In most cases, such a crossing is only apparent, since the filaments cross at different depths in the medium. Occasionally, one of the meeting filaments pierces the other. Each of the resultant segments continues its independent growth. If the obstacle is too large, the filament will bend, break at the site of the bend, and the fragments will resume their growth.

Often two or more filaments, after meeting, continue to grow parallel to each other, forming thick bacterial strands. These often consist of a mass of bacterial filaments. This does not, however, prevent the periodic departure of one or a group of filaments from the fascicle. A side ray runs to an adjoining strand, will join it or, being deflected, will run sideways. This mode of growth of a colony explains its lattice-like structure, consisting of numerous randomly arranged fascicles interconnected in complex fashion.

The existence of intercalary growth and the formation of breaks in the filaments determine the growth not only of the young peripheral portion of the colony, but also the gradual thickening of its central strands.

The growth of a colony by strands makes it appear similar to a fungal colony.

The microscope shows that the old strands, consisting of bacterial filaments, are coarse in appearance and often show branching. At the sites of curvature, most filaments turn together, but some go off sideways.

The older part of a strand usually assumes a convolute shape. This is obviously due to intercalary growth and rupture of the filaments, leading to /22 the subsequent accumulation of a bacterial mass on a limited agar surface, since that mass cannot spread out in width, the strand structure of the colony holding the bacterial cells in position.

All possible varieties of the growth of a colony of B.mycoides can be demonstrated by following, under the microscope, the peripheral part of such a colony on MPA.

One easily recognized phenomenon are the long cells at the end of the bacterial filaments. The spurious branching of the bacterial filaments is also well-defined. When placed into water, the filaments break up into groups of cells.

B.mycoides, as we will demonstrate, has several subspecies differing in the type of colony growth on MPA. This is manifest both in the structure of the peripheral part of the colony and in the structure of its center. In some cultures the B.mycoides strands run in almost a straight line, while in others they form loops, knots, etc. The central part of a colony of some variants of B.mycoides grows rather slowly, while in others the space occupied by the colony is almost completely filled by the bacterial mass.

The characteristic data of the growth of these B.mycoides subspecies on nutrient media will be presented in one of the following Sections.

The general laws of growth of a colony on MPA are the same for all variants of B.mycoides.

It should be noted specifically that the type of colony of a given B.mycoides subspecies is not determined by the quantity of inoculated material applied to the surface of the nutrient medium.

On the whole, it can be concluded that the development of a colony of B.mycoides largely depends on the existence of intercalary growth and the tendencies of the filaments to turn in a definite sense, sinistral or dextral.

The role of purely physical phenomena in the formation of colony structure can be illustrated on some simple filaments. For example, if a glass rod is placed in the path of growth of a filament of B.mycoides, the filament will creep along it. An obstacle in the form of a bit of chalk or some other body compels the filament to turn if one end rests on it, or to slide along the obstacle, if the encounter is at a sufficiently acute angle.

Pringsheim and Langer mentioned that the concentration of nutrients in the medium, especially agar, has an extremely great influence on the development of a colony of B.mycoides and on its habitus. For example, if such a colony is cultured on silica gel soaked with plain broth, then colonies with a homogeneous structure and without the characteristic offshoots of B.mycoides develop.

The appearance of the colony is affected by the amount of meat extract and other nutrients added, and especially by the concentration of the agar (Pringsheim and Langer). It may be noted that B.mycoides is very undemanding as to /23 food. On some grades of agar, this bacterium will grow even if only some carbon source is added to the medium. The small amounts of organic nitrogen in the agar itself are sufficient for its development.

The percentage of agar in the medium has an extensive effect on the type of B.mycoides colony.

In a medium containing 1 - 2% of agar, the growth of such colonies is better than on 3% agar. The coils formed by the strands are more sharply delineated on media with an elevated agar content. The coils of the bacterial strands appear to open on less concentrated media.

With small doses of nutrient in 1% agar, the colonists of B.mycoides are

atypical and ragged. The bacterial filaments are sometimes without spirals. This has been noted by many investigators (Holzmüller, Hastings and Sagen, and others).

The change in the character of the colonies on media with varying agar content is apparently based on purely physical causes. With increasing agar content, the conditions for the formation of an aqueous film on the surface of the medium become less favorable. This interferes with the growth of the colonies of B.mycoides. The colonies become more limited in size, more compact, but also denser.

Obviously, because of the capillary properties, a bacterial filament growing on a solid medium forms an aqueous film, like that noted in filamentous algae developing on agar media. When the filaments converge, the aqueous film coalesces and opposes their separation.

This adhesive force of the water no doubt is more pronounced if the aqueous film is thin, i.e., on concentrated media.

On media containing 1/8 to 3/4% agar, colonies of B.mycoides are atypical, although they do form mycelioid offshoots. Spiral coils, however, are not observed at all, and the bacterial filaments are arranged radially, with some irregular bends. The medium is rather uniformly covered by the growing mass.

On weak agar, the filaments penetrate as large agglomerates into the depth of the medium, a phenomenon that does not occur on media with higher agar concentrations. The strands growing within the medium are not circinate and are radially arranged. This again proves the role played by surface phenomena in the coiling of strands of B.mycoides. A change in nutrient concentration is /24 of considerably less influence than a change in the agar content of the medium. At low nutrient concentrations, a considerably more scanty growth of B.mycoides was noted (less dense). However, the diameter of the colony did not change.

Table 1, taken from the book by Pringsheim and Langer, gives characteristic data for the growth of colonies on media containing various percentages of agar and nutrients.

In discussing certain features of the growth of B.mycoides on agar media, Holzmüller mentioned the significance of the osmotic pressure of the medium on the development of this bacterium. For example, some cultures are able to grow on agar media that are entirely free of nutrients. In such cases, the bacteria are satisfied with the small amounts of organic compounds in the agar itself. The addition of certain amounts of NaCl to such a "starvation" agar accelerates the growth.

Holzmüller recommends the following optimum medium for B.mycoides: 100 cc water, 1% agar, 0.4% Liebig meat extract, 0.2% peptone, and 3% glucose.

For observations on the development of peripheral strands it is preferable to use poorer media, since the colonies on optimum media are dense and the offshoots are poorly delineated. However, too poor a medium will yield an atypical growth.

TABLE 1

GROWTH OF B.MYCOIDES ON VARIOUS MEDIA

Observation	Colony with 1/10 Nutrients			Colony with 1/20 Nutrients			Colony with 1/100 Nutrients		
	3% Agar	2% Agar	1% Agar	3% Agar	2% Agar	1% Agar	3% Agar	2% Agar	1% Agar
Growth of colony per hour (in μ)	100	274	322	111	208	279	69	219	375
Appearance of colonies:									
a) Macroscopically	Massive with strongly indented edges	Rather massive strands, less curved	Infrequent strands, almost radial	As on media with 1/10 normal concentration of nutrients					
b) Microscopically	Dense and greatly curved filaments		Thick filaments slightly curved						
Maximum size of colony (diameter, in cm)	2.5	9	9	3.5	9	9	2	9	9
Time required for colony to reach maximum size	2 months	3 weeks	16 days	2 months	3 weeks	3 weeks	2 months	3 weeks	3 weeks

Note: The basic medium consisted of bouillon with meat extract.

Holzmüller ran a series of experiments to study the effect of varying concentrations of agar on the habitus of B.mycoides colonies. His results were almost identical with those just presented, except that he had success with higher amounts of agar than Pringsheim and Langer. The results of the Holzmüller experiments are well worth noting. For example, he states that in the presence of 4% agar, colonies of B.mycoides are still able to grow, although slowly. In a medium with 6% agar, growth is very scanty, and at 8% agar the spores only germinate, followed by disintegration of the young cells.

Colonies of B.mycoides are usually a grayish-white tint. Very rarely they show some pigmentation, most often yellow (den Dooren de Jong, Marshall Word, Mishustin).

3. Variants of B.Mycoides Described in the Literature

The very first investigators of B.mycoides concluded, from the character of the macrocolonies, that widely dissimilar variants of this microbe must exist in nature. This assumption permitted a more detailed study of the distribution of the various subspecies in nature and led to a search for the factors responsible for the variability. The latter item, of significance for general biology, /26 attracted the attention of various authors. This research was particularly encouraged by the fact that the traits of B.mycoides make it a convenient object for studying the variability of microorganisms. Below, we give a description of several B.mycoides subspecies, isolated from natural substrates by various microbiologists, as well as of data obtained in studying the experimental variability of this organism. The papers to be discussed will be given in chronological order.

Passing to the description of various forms of B.mycoides, we first will make a brief statement as to the terminology used. In this book, the term "forms" (smooth, rugose, intermediate) will be applied to the basic groups of subspecies of substantially identical colony structure. Each form is divided into a number of "variants", for example, the smooth form has a typical smooth variant (anthracoid variant, etc.).

Each variant of B.mycoides may have ecological races, differing in physiological and morphological traits.

It can be stated that the question of the distribution of individual variants of B.mycoides in nature has never been studied in detail. There are some references to this question by individual investigators (Grundmann, Rautenshteyn, Borodulina, and others).

Holzmüller (1909) was the first to substantiate the existence of several variants of B.mycoides. He isolated four strains: α and β from the guinea-pig appendix, γ from the meal-worm stomach, and δ from soil.

These cultures differed in certain traits from the B.mycoides of Flügge obtained from the Krahl collection. The traits of all these cultures were constant and persisted for a long time.

A medium of the following composition was selected for the study of B.mycoides variants: 1% peptone, 0.8% Liebig extract, 0.5% NaCl. The agar media contained 1% agar-agar and the gelatin media, 8% gelatin .

The principal distinctive features of the variants studied by Holzmüller are given below.

B.mycoides α. On MPA, the strands are more or less straight, with numerous branchings (Fig.4). In a 3-day culture, the bacterial filaments of the central part of the colony already contain spores. In a 14-day culture, there are /27 only spores released from the cells but still lying in the same arrangement as the filaments, so that the character of the colony remains unchanged.

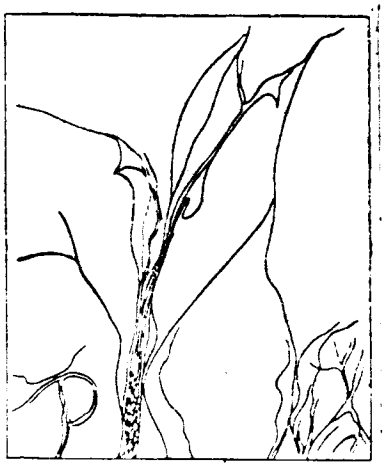


Fig.4 B.Mycoides α (according to Holzmüller)

The cells with spores have less acute angles than the cells without spores. The size of the cells is 2.83 - 3.68 μ in length and 1.45 - 1.87 μ in width.

The motility of the bacteria is slight. The color of the flagella is likewise negative. The rods are surrounded by a capsule, which is clearly visible when the preparation is stained with Methylene Blue.

The mature spores are not covered by the membrane of the parent cell. The growth of the spores is predominantly polar. The spore capsule remains for quite some time at the tip of the rod from which it grows. Sometimes the growth of the spores is lateral. Still less often it is from both ends. The ratio of these modes of growth is 10:2:1.

After 36 hours, a white sediment forms in the broth. When shaken, this precipitates in the form of small plates. The liquid becomes turbid, apparently from separation of individual cells. No films are formed on the surface of the medium.

Without access to oxygen, the growth is far slower. In this case, the type

of growth is as usual, but no sporulation is noted.

B.mycoides β. On MPA, the bacterial filaments are more circinate than in the strain α. The coils are small, run in different directions, and are sometimes spiral (Fig.5). The filaments in the colony turn in no definite sense. Under the microscope, the filaments appear to form a tangled web. This results

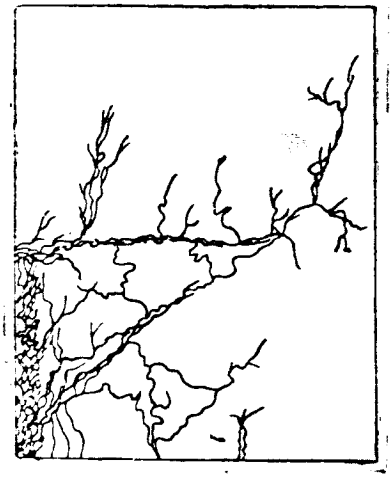


Fig.5 B.Mycoides β (according to Holzmüller)

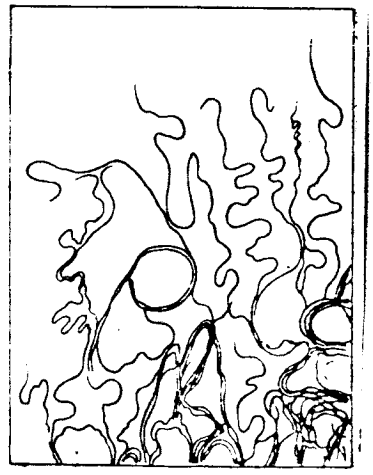


Fig.6 B.Mycoides γ (according to Holzmüller)

from the fact that the filaments do not grow in parallel order, but in intricate patterns. The inner part of the colony consists of circinate strands. Cell size: length 3.47 - 4.92 μ, width 1.2 - 1.4 μ. The corners of the cells are subrounded.

The spores are larger than in the strain α.

The growth of the spores is most often equatorial, less often polar. The ratio of these modes is 6:1. Before germination, the spore shows swelling.

The rod is often saccate at the site of the spore.

B.mycoides γ. On MPA, the bacterial strands are always thin and beautifully curved. They are spaced rather far apart, so that the bacterial mass in a colony appears uniformly scattered. Under the microscope, the coils show as being far larger and not as regular as in B.mycoides β (Fig.6). /28

The length of a rod is 2.31 - 4.62 μ and the width, 1.2 - 2.85 μ. The spores in the mature state are often surrounded by the membrane of the parent cell. Before germination, they show strong swelling. Germination is usually in radial or equatorial direction, from a bend of the germinating rod. The ratio of these modes is 4:1.

B.mycoides δ. On MPA, this form grows at an exceptional rate. The increase in length of the bacterial filaments may be as much as 500 μ per hour. The sense of rotation of the strands is not definite. Most often, however, it is counterclockwise. At some points along the peripheral strands, nodule-like formations are noted (Fig.7), especially at the branching points. The strand leaving a nodule forms a complex coil at a certain distance. At the edge of a colony, spirally coiled bacterial filaments may also be observed.

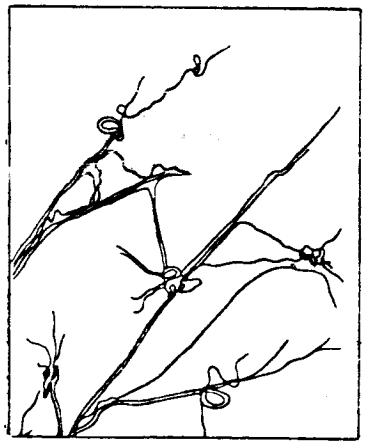


Fig.7 B.Mycoides δ (according to Holzmüller)

The rods are 2.08 - 4.17 μ long and 0.87 - 1.39 μ wide. The spores are usually covered by the membrane of the parent cell. The sporiferous cells are distended at the site of the spore. For every 10 spores with polar germination there are six with equatorial germination (in the form of a curved rod) and one growing from both poles.

B.mycoides Flügge (Krahl). On agar medium, the colony grows slowly. The strands exhibit a combination of elements of type β and type δ. /29

The membrane of the parent cell does not drop off the spore for a rather long time. The germination of the spore is always polar.

It is only approximately possible to say what variants of B.mycoides were studied by Holzmüller, since he observed the character of the strands only in the initial stages of colony growth. As we will show below, the structure of an old colony yields considerably more information than the structure of the strands in a young colony.

However, one can assume that the types α, γ, and δ of B.mycoides belong to the rugose variants of these microbes, while B.mycoides β must apparently be assigned to the smooth type.

In 1924, Perlberger published a paper devoted mainly to the fermentative properties of various cultures of B.mycoides, obtained from the Pribram collection. These also included cultures of bacteria, incorrectly described as B.mycoides by several authors.

All the bacteria used in this work were gram-positive. From the cultures available to him, Perlberger first distinguished a group containing no carbohydrate-decomposing enzymes.

These include B.mycoides corallinus Hefferan, B.mycoides roseus Scholl and B.mycoides bruneus γ. All these forms give a red color. The first two (according to Hefferan) are of the nonsporulating type, and were therefore assigned to the group of B.prodigiosum. The author has demonstrated that this group of bacteria does sporulate.

From their cultural properties and their behavior toward carbohydrates it is obvious that all these bacteria, just like the yellow B.mycoides luteus Schnürer, have no relationship at all to B.mycoides Flügge. They were identified as belonging to this genus because of their ability to produce offshoots somewhat resembling B.mycoides Flügge on dense media.

Forms closer to the typical B.mycoides caused acid formation on sugars. /32
Perlberger believes that B.mycoides does not ferment lactose.

He considers Holzmüller's differing opinion incorrect, since this was a conclusion based on the coagulation of milk.

One of these forms, N.mycoides var. ovoaethylicus Wagner, produces gas when grown on sugars, and is apparently far from being related to B.mycoides Flügge.

The various strains of B.mycoides behave differently toward the glucosides. Table 2 shows the sugar reaction of the bacteria studied by Perlberger. As indicated there, he classified these bacteria in some detail but primarily with respect to their growth on potato media: Group I comprises cultures giving a red pigment, Group II those giving a yellow pigment, Group III those giving no pigment, Group IV those with feeble or scanty growth, and Group V those giving a slimy growth on potato and forming gas from carbohydrates.

TABLE 2

BIOCHEMICAL PROPERTIES OF VARIOUS CULTURES OF B.MYCOIDES (ACCORDING TO PERLBERGER)

Group	Name of Bacteria	Dimensions of Rods, μ	Dimensions of Spores, μ	Motility	Growth on Sugar Agar	Growth on Slant Gelatin	Arabinose a	Arabinose g	Dextrose a	Dextrose g	Levulose a	Levulose g	Mannose a	Mannose g	Galactose a	Galactose g	Saccharose a	Saccharose g	Maltose a	Maltose g	Lactose Mannite Dulcite a	Lactose Mannite Dulcite g	Raffinose a	Raffinose g	Methyl glucoside a	Methyl glucoside g	Amygdalin a	Amygdalin g	Arbutin a	Arbutin g	Salicin a	Salicin g	Dextrin a	Dextrin g
I Red pigment on potato media	*Bac. mycoides corralinus* Hefferan	0.76—1.14 1.63—3.1	0.55	—	Glistening red film w/o offshoots	Liquefies in 3 wks	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bac. mycoides roseus Scholl.	0.62 0.72—1.02	0.6—1.14	—	Dry brick-red deposit	Not gelatinolytic	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Bac. mycoides bruneus γ	0.41—0.76 1.7	Round	—	Dry brick-red deposit	Same	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
II Yellow film on potato	*Bac. mycoides lut.* Schnürer	1.53—1.72 3.06—4.02	1.84×1.52	+	Glistening deposit	Slightly gelatinolytic	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
III Colorless film on potato	*Bac. mycoides* Holzmüller	0.76 2.24—3.5	0.9—1.0 1.3—1.5	—	Gray-white deposit with numerous shoots	Strongly gelatinolytic	—	—	+	—	+	—	—	—	—	—	—	—	+	—	—	—	+	—	—	—	—	—	+	—	+	—	+	—
	Bac. mycoides Holzmüller	1.02 4.59—5.52	1.25 1.53		Same	Same	—	—	+	—	+	—	—	—	—	—	—	—	+	—	—	—	+	—	—	—	—	—	+	—	+	—	+	—
	Bac. mycoides Flügge № 3	1.2 7.06—4.5	1.14—1.4 1.5—1.8	+	"	Gelatinolytic	—	—	+	—	+	—	—	—	—	—	—	—	+	—	—	—	±	—	—	—	—	—	+	—	+	—	+	—
	Bac. mycoides Flügge № 4	1.22 3.06—4.5	1.02 1.5		"	"	—	—	+	—	+	—	±	—	—	—	+	—	+	—	—	—	+	—	—	—	—	—	—	—	—	—	+	—
	Bac. mycoides Gersbach	1.53 2.82—6	0.76 1.02	—	"	"	—	—	+	—	+	—	±	—	+	—	+	—	+	—	—	—	+	—	—	—	—	—	+	—	+	—	+	—
IV Scanty or slight growth on potato	*Bac. mazun* Gruber	—	0.8 1.62	+			—	—	+	—	+	—	—	—	+	—	—	—	+	—	—	—	+	—	—	—	—	—	+	—	+	—	+	—
V Slimy growth on potato Produces gas on carbohydrate	*Bac. mycoides ovoaethylicus* Wagner	0.75 2.29—3.6	0.8 1.52	+	Grayish deposit with slimy strands	Slightly gelatinolytic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	Lactose + + Mannite + − Dulcite − −		+	+	+	+	—	+	+	+	+	+	+	+

the width, the lower the length. 2. In the columns characterizing the behavior toward sugars, the letter "a" denotes acid formation, the letter "g" gas production.

Obviously, only the third and perhaps also the fourth group of these microorganisms belong to the group of B.mycoides Flügge. From Perlberger's data, it is still possible to conclude that the individual cultures of B.mycoides differ in biochemical properties.

Nyberg (1927, 1929) studied several cultures of B.mycoides. He was interested in the question of possible dissociants of this organism. Using the usual nutrient media for the multiplication of B.mycoides, he was able to produce the various subspecies in the culture by transfer culture on meat-peptone media.

Among the colonies obtained by him on aged cultures, were some resembling typical colonies and some similar to the well-known colonies of B.vulgatus. In some cases, the variants isolated differed in stable traits, while in others they reverted to the original variants or even gave new variants.

Thus, Nyberg showed the possibility of obtaining, under laboratory conditions, from the typical form of B.mycoides, several variants whose colony character remotely resembled the original form of this microorganism. The "d" forms described by him were very similar to the smooth variants of B.mycoides obtained by other authors (cf. infra).

Nyberg believed that the typical form of B.mycoides was the hybrid form, from which new types are obtained by the usual fission. This view is of course completely unfounded.

Physiologically, the smooth and rugose forms differ in no way. /33

The question of dissociation in B.mycoides was treated by Oesterle and Stahl (1930). They attempted to accelerate the phenomenon of dissociation in B.mycoides by culturing it under unfavorable conditions.

Oesterle used a soil extract (putrefying suspension) and exposed the broth culture to sunlight or to ultraviolet rays.

Prolonged culturing of these bacteria in the soil extract led to the disappearance of typical cells from the medium. Round and vacuolate cells were present in the preparation. When such cultures were inoculated on MPA, atypical transparent brownish colonies, which later sprouted, were developed. Many of them resembled colonies of the potato bacillus. The cells were 0.5 - 0.6 μ wide and 0.5 - 1.5 μ long. After several passages on broth they reverted from the newly formed cultures to the typical form of B.mycoides.

Other cultures maintained a growth similar to B.mesentericus. These were termed smooth forms.

A comparison of several properties of the ordinary (R) and smooth (S) forms is given in the accompanying Table.

Oesterle obtained a roughly analogous picture by prolonged cultivation of B.mycoides at various temperatures on an extract from putrefying suspensions. Transfer cultures from the old culture yielded smooth colonies. On the next /34 transfer to fresh media, the atypical forms reverted to a form of the original

Medium	Rugose Form	Smooth Form
MPA	Colonies with numerous shoots, difficult to pick up with a loop	Fatty colony with more even edges. The mass is easily picked up with a loop
MPA	Heavy film, often settling to the bottom of the test tube, leaving the broth transparent	Thin film. Distinct turbidity of the broth
Microscopic picture	Long chains of gram-positive rods	Single or paired bacilli. Gram-positive

type.

The exposure of a broth culture to sunlight led to a rapid change of typical cells. Cultures exposed for two days in succession yielded a filtrable form of B.mycoides. Culturing of the filtrate itself, on a solid medium, gave a negative result. Conversely, when the filtrate was placed in broth and left there for one week, a subsequent inoculation led to the appearance of slimy grayish colonies with smooth edges, consisting of cocci (0.4 - 0.5 μ). In a number of cases almost transparent colonies, likewise consisting of cocci (0.35 - 0.6 μ), were obtained.

In transfer cultures on broth and MPA, these forms produced colonies which on aging gave shoots somewhat resembling those of B.mycoides. In the latter case, the preparation contained not only round cells but also gram-positive bacilli (0.7 × 2.5 μ).

A typical B.mycoides colony could also be obtained from an old broth culture of one of these variants.

Hence, the author concluded that B.mycoides cultures, modified in one way or the other, revert to their original form. In particular, the filtrable form of B.mycoides does revert to the original form.

Under the action of ultraviolet light, the rods of B.mycoides were converted into round and filamentous formations. However, when these forms of cells were inoculated on agar, they gave typical colonies.

Many of the modified forms were more or less constant and produced colonies of a specific form on agar. Their description is given by Table 3, compiled by us from the Oesterle data.

In principle, Oesterle recognized the possibility of a conversion of atypical experimental variants into a typical form of B.mycoides. This rever-

sion can take place suddenly or may have a series of intermediate forms.

We will not go into the details of these transformations.

The co-author of Oesterle, Stahl, induced the dissociation of B.mycoides by an addition of various chemical substances, NaCl, Na_2CO_3, etc., to the medium.

The variants obtained by him, which are entirely identical with those described in Table 3, comprised forms giving colonies that greatly resembled B.offusus, B.olfactorius, and B.nanus.

A critical analysis of the work of Oesterle and Stahl indicates that some of their conclusions are erroneous. In one of the following Chapters we will come back to their work with respect to the morphological variations of the B.mycoides cell. Here, we will merely discuss statements on the phenomenon /35 of dissociation in this microorganism.

There is no doubt that these authors correctly noted the presence of smooth and anthracoidal variants among the B.mycoides (form 6 and 7). However, none of the other variants found were ever confirmed by later authors; they must be considered as having been due to methodological errors, mainly to contamination.

The insufficient purification of cultures obviously explains the easy reversion of atypical forms to the original variant of B.mycoides. We know today that, while the rugose form of B.mycoides passes with relative ease into the smooth form, the reverse transformation can generally not be obtained.

The work of Stapp and Zycha (1931) on the variability of B.mycoides deserves attention. The working cultures for the experiments were isolated by means of a micromanipulator.

The following media were used for culturing B.mycoides:

D: peptone, 1.2%; Liebig extract 0.8%; dextrose 1%; agar 2%.
K: potato agar, prepared by boiling a cut potato with double the quantity of water. In all, 2% of agar was added to the medium.

The original cultures for the experiments were isolated from soil. Of 13 isolated cultures, three were assigned to the dextral and two to the sinistral type; the remainder was diffusely distributed through the medium and showed no distinct rotation of the strands.

The experiments demonstrated the spontaneous occurrence of the "smooth" forms, from the ordinary rugose forms of B.mycoides.

On agar D medium, the smooth races gave brownish-white colonies with a slightly granular surface. These colonies had coalescent shoots and only slightly resembled the typical colonies of B.mycoides.

On D broth, the rugose forms formed myceloidal floccular sediments. In test tubes inoculated with smooth races, a slight turbidity and uniform sediment were observed.

TABLE 3

DISTINCTIVE PROPERTIES OF VARIOUS VARIANTS OF B.MYCOIDES,
(ACCORDING TO DATA OF OESTERLE AND STAHL)

Trait	Form No. 1	Form No. 2	Form No. 3	Form No. 4	Form No. 5	Form No. 6	Form No. 7
Type	Typical form of B.mycoides	Gram-negative bacillar gonidia. Obtained after breakdown of typical forms of rods and sometimes from filtrable forms	Yellow intermediate bacillary form. Obtained from form No. 2	Gram-negative round cells obtained from filtrable forms	Red form giving round cells. Obtained from filtrable forms	Smooth form of B.mycoides obtained from typical B.mycoides, from gram-negative round and other forms	Form recalling B. anthracis. Obtained from typical form under ultraviolet irradiation, and also from filtrable forms
Growth on MPA	Description in text	Grayish small colonies moist and glistening; edge of colony delicate and hardly visible	Lemon yellow fatty colonies, glistening at first. Later assumes a light-brown tinge, especially toward edge	Dirty white, copiously growing colonies with no definitely demarcated edge. On buildup, forms shoots	Orange-red colonies with even edges	Irregular digitate glistening colonies;	Round colonies with greasy luster. Edge resembles B. anthrax colony;
Microbiological picture	Same	Rods 0.75 × 0.75 - 1.5μ	Rods 0.35 - 0.4μ × × 0.75 - 1.5μ	Cocci 0.5 - 0.7μ	Cocci and staphylococci 0.5 - 0.75μ	Rods 0.5 - 0.75μ × ×1.5 - 3.5μ	Rods 0.5 - 0.75μ × × 4.5 - 5.0μ
Gram stains	Same	Negative	Negative	Negative	Positive	Indeterminate	Positive
Motility	Same	Slight	Slight	Immobile	Immobile	Mobile	Immobile
Behavior toward gelatin	Same	Not gelatinolytic	Gelatinolytic	Not gelatinolytic	Not gelatinolytic	Gelatinolytic	Gelatinolytic
Grows on broth	Same	Forms thin pellicle, slight turbidity of medium, and slight sediment	Uniform turbidity, thin pellicle, and slimy sediment	Strong turbidity and abundant sediment	Slight turbidity. Has floccose sediment	Uniform turbidity and thin pellicle. Sediment, floccose	No turbidity nor pellicle. Has slimy sediment
Grows on potato	Same	Originally grayish pellicle. Later acquires brownish tinge	Yellow abundant dry pellicle	At first glistening thick pellicle which then dries out	Thin orange pellicle, rapidly drying out	Yellowish-white dull pellicle	Dry gray-white pellicle
Grows on agar	Same	On agar the form may persist for a long time, capable of passing into forms Nos. 3 or 4	Passes into round forms in a few weeks	Form rather constant. May pass into smooth forms	Intermediate form in passage of other variants into smooth forms	Rather constant form. May pass into R-form	Passage into R-form possible. Gives asporogenous cultures

Formation of the S-forms was observed on prolonged cultivation of B.mycoides on D broth. These variants were easily discovered on subsequent transfer cultures on solid media. On agar cultures, no fission of smooth forms was obtained. Only a single case of reversion of an S-type to the R-type was observed, and that in a highly variable strain of B.mycoides.

Stapp and Zycha obtained smooth variants from many cultures of B.mycoides, after 30 - 104 days of cultivation in a liquid medium.

In studying soil microflora it has been possible to isolate three smooth /38 variants directly from soil.

In a hanging drop, the S- and R-forms give a different type of growth. In the R-variants, the cells remain connected to each other and form long filaments. In the S-forms, only short filaments are formed, which then collect in the center of the drop and send off weak shoots toward the sides.

A still more detailed investigation on the dissociation of B.mycoides was performed by the American investigator Lewis (1932).

This researcher experimented with three pure cultures of B.mycoides, isolated by means of a micromanipulator. A fourth culture was obtained from a collection.

All these cultures of B.mycoides were assigned to the smooth variant, although there were some differences in their growth on a solid medium. One culture was sinistral, the other three dextral.

At the beginning of his report, Lewis gave details on the conditions corresponding to the manifestation of dissociation.

He emphasizes that, in the opinion of a number of authors, dissociation proceeds better in liquid media. His work completely confirms this conclusion. If B.mycoides is cultured on broth and is passaged every day, then the original passages on MPA from this medium show no atypical forms.

At later stages, ever more often, in passage to the solid medium, variants of B.mycoides resembling B.vulgatus and B.subtilis are encountered. In the liquid medium, these variants gave a growth typical for the smooth variants of B.mycoides. However, the microscopic picture remains constant.

Lewis considers that, in rapid multiplication promoted by frequent passages, the number of variable cells increases.

Nevertheless, even frequent transfer cultures on MPA do not lead to the appearance of dissociated forms. The cultures of B.mycoides remain practically stable.

In giant colonies of B.mycoides, variability in certain sectors or along the edges of the colony can sometimes be observed. This phenomenon was also noted by Nyberg.

Occasionally, daughter colonies are encountered on colonies of 2 - 3 weeks growth. Below, we will further discuss the nature of these colonies. Apparently, they are not identical with the so-called G-colonies, since the size of the cells composing these colonies does not differ from that of normal cells. An inoculation of cells from daughter colonies will again give typical colonies of B.mycoides.

The view that unfavorable growth conditions promote the phenomenon of /39
dissociation is generally accepted. Lewis pointed out that most authors have not paid sufficient attention to the dissociation of microorganisms in normal media. At the same time, for instance from the work by Hadley (1931), it is clear that the transformation of a Shiga bacillus into the G-type was equally successful in media with and without LiCl. There are strong indications that some types of variants cannot be obtained on certain media, without the addition of certain substances.

In his work, Lewis verified the effect of several physical and chemical factors on the phenomenon of dissociation.

He found that an elevated temperature (30° C) promotes an increase in the number of dissociants.

In media of different pH, ranging from 5.4 to 8.8, no substantial differences were noted.

B.mycoides grows well on dilute media. Dissociation was observed in media diluted 5 - 10 times, but was not noted in media diluted 20 times. This law was confirmed in experiments lasting as long as four months.

In milk, the phenomenon of dissociation was weaker than in broth. Dissociation was also noted in a liquid casein medium, yeast agar, gelatin, 1% peptone or meat extract, diluted with water.

The experiments permitted the conclusion that the dilution of a medium does not favor the appearance of dissociants.

In earlier work (Nadson and Adamovich), the promoting effect of metabolites on the appearance of atypical forms of B.mycoides was noted. Later, this was also found by Nyberg.

Lewis admixed the nutrient media with filtrates of several old cultures (Pseudomonas fluorescens, Serratia marcescens, and B.subtilis). Not one of the strains of B.mycoides was able to grow in a medium with 5% filtrate of Ps.fluorescens and 25% filtrate of a culture of B.subtilis. At lower concentrations of the metabolites, the dissociation of B.mycoides set in no sooner than on the control medium, and usually much later.

A similar result was obtained in experiments with filtrates of other cultures. The addition of peptone and meat extract to the media intensified the growth and the development of dissociants.

Various concentrations of NaCl, LiCl, brucine sulfate, morphine sulfate,

cocaine hydrochloride, and corrosive sublimate were added to liquid media. Concentrations of these substances that were sufficient to inhibit the development of B.mycoides, also prevented the appearance of dissociants.

Lewis stayed away from generalizations but did indicate that dissociation /40 in B.mycoides, contrary to the statements by other authors, cannot be considered as due to a pathological process nor as a result of disorders of physiological metabolism. Dissociation is a normal process.

Lewis gave detailed descriptions of the forms of B.mycoides isolated by him, in studying the phenomenon of dissociation. We present here only the principal data on their characterization.

Variant I. This variant resembles the original strain. The bacterial strands have no coils and the side branches proceed at right or acute angles. In addition, this type of colony does not grow so far into the agar, and the strands have a clearly visible structure. This variant maintained its distinctive traits for two years of transfer culturing on solid media, but rapidly passed over into other variants when cultured on fluid media.

The first variant developed less often than the others, but was encountered repeatedly. Its isolation was easiest by inoculating a culture of B.mycoides from milk into a solid nutrient medium. In our opinion, this first variant is one of the subspecies of the rugose form of B.mycoides.

Variant II. This variant resembles only partially the original culture in character of the bacterial strands, but does not grow into the medium and does not form such long strands.

A microscopic study of the strands on the peripheral part of the colony shows extensive straightening of the individual bacterial filaments.

Variant III. This variant is similar to the preceding, but the contours of its colonies are more blurred and less symmetric. The young colonies resemble the original culture, but do not grow into the nutrient medium.

Judging from these descriptions, there is no substantial difference between Cultures II and III, especially in the microscopic picture of the strands. It seems that both these variants belong to the S-form of B.mycoides.

Variant IV. This variant forms dense cloudy colonies. The peripheral strands of the colony have an effuse structure and a considerable width. The colonies are easily removed from the nutrient medium, thus giving the effect characteristic for the smooth forms of B.mycoides.

Variant IV resembles the δ-type of Holzmüller.

Variant V. This variant is very similar to the preceding type, except for the tendency to form miniature colonies with delicate strands, resembling the structure of the strands of type IV.

These colonies are similar in appearance to B.subtilis, described by /41

Soule (1927), and should be classified with the smooth variants of B.mycoides.

Variant VI. This variant is characterized by the complete absence of peripheral shoots. The old colonies are surrounded by something like fimbria, easily distinguishable in structure from the shoots of the preceding types. The edge of the colony, just like the colony itself, has an effuse topography, characteristic of B.subtilis and B.vulgatus.

We classify this variant as "anthracoid".

Variant VII. This variant greatly resembles the preceding, but differs from it in the character of growth. The young colonies have a form characteristic for the smooth forms of B.mycoides with almost integral edges. The short strands are characteristically circinate.

Lewis gave a supplementary description of several intermediate types, whose characterization we will omit here.

It seems that all of the Lewis variants can be classified with two main forms, smooth and rugose. In fluid media, dissociation proceeds generally from the rugose variant to the smooth. The former, besides other differences, is also characterized by a strong tendency to grow into the nutrient medium and by considerable arborescence of its strands.

Most types of B.mycoides described by Lewis were also mentioned by other authors. Some variants, obtained by Oesterle and Stahl, were not found by Lewis. Chromogenic and coccal stable variants are completely absent, as are also gram-negative rod-like forms.

Lewis did not observe a reversion of the smooth forms of B.mycoides to the original, even under the special culture conditions recommended for this purpose by other authors.

Variants of the rugose type gave a reversion to the original type when cultured on standard media at room temperature.

Changes in colony structure had no substantial effect on the physiological properties of the variants of B.mycoides.

Figures 8 - 16 are photographs of several of the most typical variants of B.mycoides described by Lewis.

In 1933, Kushnarev described dissociation in B.mycoides under the action of low temperatures (from 3° to 12° C). Spores of B.mycoides emulsified in distilled water were cooled, and cultures on MPA were periodically prepared from an aqueous slurry.

A brief exposure to low temperatures made no appreciable changes in the /44
original cultures. Later, however, a number of modified forms were obtained. Kushnarev divided them into two groups.

Group I: Mutants, differing sharply from the original forms of B.mycoides,

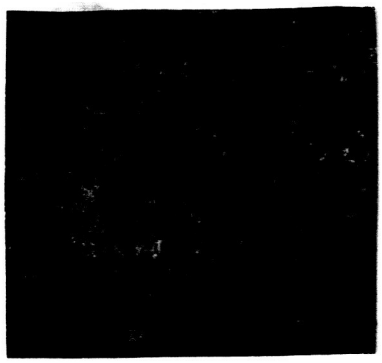


Fig.8 Colony of Rugose Form of B.Mycoides (according to Lewis)

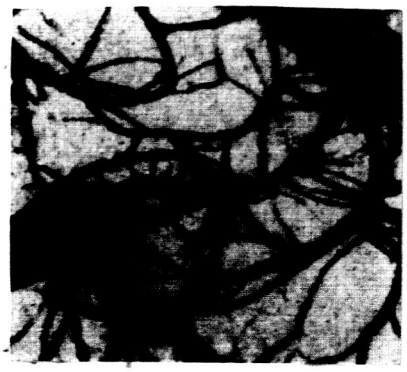


Fig.9 Sector of Colony of Rugose Form of B.Mycoides (according to Lewis)

Fig.10 Structure of Distinctly Visible Variant of Rugose Form of B.Mycoides (according to Lewis)

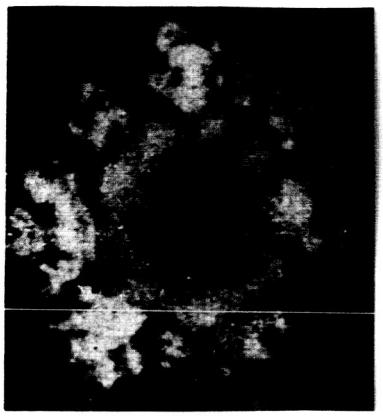


Fig.11 Colony of Smooth Form of B.Mycoides (according to Lewis)

Fig.12 Edge of Colony of One Variant of Smooth Form of B.Mycoides (according to Lewis)

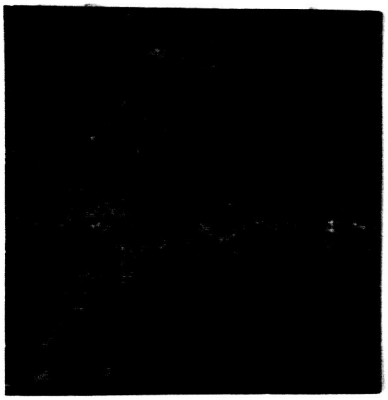


Fig.13 Edge of Colony of Another Variant of Smooth Form of B.Mycoides (according to Lewis)

Fig.14 Structure of Colony of One Smooth Variant of B.Mycoides (according to Lewis)

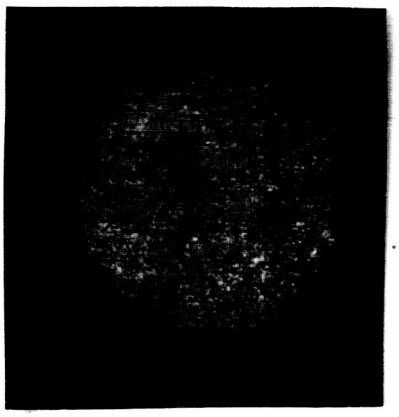


Fig.15 Colony of Anthracoid Form of B.Mycoides (according to Lewis)

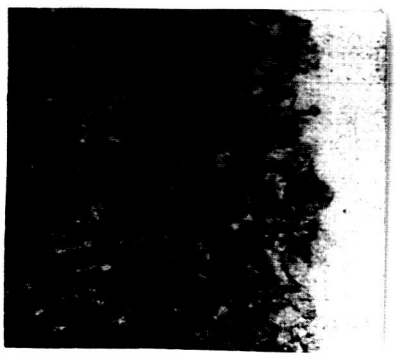


Fig.16 Edge of Colony of Anthracoid Form of B.Mycoides (according to Lewis)

in the absence of spores, motility, slow gelatinolysis, character of growth on MPA, and behavior to sugars.

On MPA, bacteria of this group give a mycelioid smooth growth; the bacterial pellicle is easily removed. The author gives no more detailed description of the type of colonies on this or other media.

On potato, a delicate and slightly pigmented pellicle is formed.

On MPA, a thin pellicle forms generally; at times, growth proceeds without pellicle formation.

All traits of the mutants are preserved in the progeny.

Group II: Modifications rapidly losing the acquired characteristics when cultured on ordinary media. In cultural and biochemical properties, they differ little from the original strains, but the colonies do not show the typical mycelioid growth.

Kushnarev noted that some variants of B.mycoides, in their morphological and biochemical traits were entirely identical with a typical form of this bacterium, but that most of them differed substantially from the original cultures.

Certain lipoidal inclusions are often formed in the cells of new variants.

Table 4 gives a more detailed compilation of the distinguishing characteristics of Groups I and II.

In giving a critical evaluation of Kushnarev's work, we must emphasize that, in several careful studies on dissociation, we were unable to detect such substantial differences in the structure of the B.mycoides cell and its enzyme complex. Nevertheless, it is likely that, aside from the inherited variability of this bacterium, also variations of a modification type exist.

In 1935, two papers devoted to the pleomorphism of B.mycoides were published, one by Schmidt and the other by den Dooren de Jong.

Schmidt studied the variability of six cultures of the anthrax bacillus and two cultures of B.mycoides. Various media were used to grow these microorganisms, including sugar agar, potato, sterile manure, plain broth, etc. The bacteria were held 8 - 12 weeks on these media, and were then transferred to agar media. Here, two types of colonies were formed. The first medium had the usual characteristic colonies. In the second type, the colonies were colorless and slimy, without typical shoots (smooth variant). In the microscopic picture, there were no major differences between the rods of B.mycoides of the two types. In the smooth variant, only less spores were formed. /46

Thus, Schmidt's work again confirms the coexistence of the above smooth forms with the rugose variants of B.mycoides.

Certain new factors were introduced by den Dooren de Jong in the characterization of B.mycoides. She was able to isolate B.mycoides from soils of Den-

TABLE 4

PROPERTIES OF THE VARIANTS OF B.MYCOIDES DESCRIBED BY KUSHNAREV

Culture	Size of Rod, μ	Size of Spores, μ	Motility	Gram Stain	Medium					Behavior of Sugars				
					Gelatin	MPA	Potato	Plain Broth	Milk	Saccharose	Galactose	Maltose	Lactose	Glucose
Original	0.5 × 3-6	0.75×1-2	-	+	Liquefies rapidly	Myceli-oid	Brown coating	Forms pellicle	No change	+	-	-	-	+
Group I 1	0.5 × 3-6	-	+	+	Slowly lique-fies	Slimy	Pink coating	No pellicle	Same	-	-	-	-	+
2	0.5 × 3-4	-	+	+	Same	Rough, Slimy edge	Same	Delicate pellicle	"	-	-	-[1]	-	-
5	0.5 × 3-4	-	+	+	"	Mucoid	"	Like No.1	"	-	-	-	-	-
7	0.5 × 3-4	-	+	+	"	Rough	"	Like No.2	"	-	-	-	-	-
8	0.2 × 5-6	-	+	+	"	Deli-cate	Slimy	Like No.2	"	-	+	-	+	+
Group II 3	0.5 × 4-6	1-2	-	+	Lique-fies	Smooth colonies easily removed	Rugose growth	No pellicle	"	+	-	-	-	+
4	0.5 × 3-6	0.75	-	+	Same	Same	Same	Delicate pellicle	"	+	-	-	-	+[1]
6	0.5 × 4-6	0.5×1-2	-	+	"	"	"	No pellicle	"	+	-	-	-	+

[1] Gas evolved

mark and Germany.

The individual cultures of B.mycoides had a pronounced lemon tinge. We succeeded in isolating a similar form from tundra soil of the vicinity of Igarka. A culture giving a yellow pigment was also isolated by Marshall Word from the waters of the Thames River.

In the yellow forms of B.mycoides, the pigment is deposited as a granular mass outside the cells.

den Dooren de Jong was occasionally able to isolate the smooth forms of B.mycoides in transfer cultures from bouillon to agar. Some strains, however, gave no separation of the smooth forms.

Smooth variants were separated both from unpigmented and yellow cultures of B.mycoides. From the pigmented cultures, she was able to obtain white mutants with strongly fixed hereditary traits.

The yellow and white variants of B.mycoides yielded asporogenic cultures.

In discussing the characteristics of the S-variants, den Dooren de Jong noted that some of these do not branch at all. This type of colony evidently corresponds to those we consider as being anthracoid. In the author's opinion, B.undulatus greatly resembles one of the variants of B.mycoides.

A study of the reversion of the mutated forms to the original forms induced den Dooren de Jong to establish the following propositions:

1. The white variants of yellow strains do not pass into the earlier forms.

2. Asporogenic forms may revert to the original forms.

3. A passage of smooth variants into rugose variants is observed only in exceptional cases. The character of the strands however is not entirely identical with the original forms.

den Dooren de Jong doubted the conclusions by Oesterle and Stahl. Her attempts to obtain filtrable forms, red colonies of B.mycoides, and the like, all failed.

Borodulina (1935) stated that, under the influence of the metabolic pro- /47
ducts of actinomycetes, B.mycoides may form asporogenic subspecies that grow poorly on medium suitable for the original culture (mineral-peptone water). The introduction of glucose into the medium renders it suitable for the development of the mutated variant. On mineral-peptone agar, this variant forms smooth transparent colonies, with branching inside the agar. These forms evidently must be classified with some smooth variants of B.mycoides.

Rautenshteyn (1937) isolated 25 typical cultures of B.mycoides from various sources (soil and water). In accordance with their cultural, morphological, and physiological traits, these cultures can be divided into two sharply differ-

ent groups. Representatives of the first were found in all soils examined, those of the second only in soils of the southern regions.

Under laboratory conditions, from cultures obtained by the method of "old broth cultures", i.e., by prolonged culture in flasks with MPB (meat-peptone bouillon) followed by transfer to solid media, a number of variants were isolated.

Rautenshteyn described these in a paper published in 1946. He divided the entire collection of his cultures into the following groups:

First variant. On MPA, this variant forms flat colonies with compact central part, from which long spirals extend. The edge of the colony is formed by thick filaments. The colonies are easily removed from the agar, and the bacterial mass is readily emulsified. This is the usual smooth form of B.mycoides.

Second variant. This variant outwardly resembles the rugose form of B.mycoides, but differs from it in internal structure of the colonies, namely, in thickness of the filaments forming the colony and in their mutual position. The colonies are easily removed from the agar.

Apparently, this variant is close to the smooth form of B.mycoides.

Third variant. The colonies are compact with entirely smooth or festooned edges. The colonies do not give the peripheral shoots characteristic of B.mycoides, or such shoots occur at other spots.

This variant is close to our designation of "anthracoid" variant.

Fourth variant. This subspecies forms mycelioid colonies resembling the rugose form, but the bacterial mass is easily removed from the agar. The surface of the colony has glistening nodules formed by peculiarly circinate bacterial strands. The author calls this variant "iridescent". In our opinion, it is close to the rugose form of B.mycoides. /48

Fifth variant. This variant forms colonies of the mesentericus-like type, with the rugose surface in the form of a mesentery. The edge of the colony is festooned and occasionally shows sprouting of small coils.

Sixth variant. This subspecies forms small colonies with a smooth surface and indented edges. The colonies consist of what might be called "coalescent nodules", and are removed from the agar only with difficulty. They are unstable and easily revert to the original form.

Frequent transfers and elevated culture temperature promoted an accelerated production of dissociants. It was harder to induce dissociation in southern cultures of B.mycoides than in northern cultures.

Variants of B.mycoides directly isolated from the soil are very close in cultural and morphological traits to those obtained experimentally in the laboratory, and are sometimes completely indistinguishable from them.

A study of the physiological properties of the original cultures and their experimental variants showed:

a) Differing behavior toward certain carbohydrates (saccharose, dextrin, starch) and gelatin, and differing intensity of ammonification, etc., in the subspecies and the original forms.

b) Frequent failure of concomitant variation of cultural traits of B.mycoides and changes in physiological properties. The cultural and morphological variability does not always run parallel to the variability of the biochemical properties.

c) Individual cultures that do not differ culturally or morphologically may still differ in certain physiological traits.

Nakhimovskaya (1937) studied the variability of colonies of B.mycoides under the influence of actinomyces antagonists. She observed such varied colonies: a) on the boundary of the "sterile wave", surrounding a colony of the actinomycetes antagonistic to B.mycoides; b) on agar plates, to which a filtrate from a culture of actinomycetes with toxic substances, separated in the substrate, had been added. A comparison of these modified colonies with normal colonies of B.mycoides permits a classification into three groups.

The first group comprises leathery, rugose, dense, mesenteroid colonies with scarcely perceptible, very short, and what might be called retracted, shoots. This type of colonies appeared either on the boundary of the "sterile zone" in various cultures of actinomycetes, or on agar plates with a medium containing filtrates of certain Actinomycetaceae.

The second group contains small colonies sometimes up to 5 mm in diameter, strongly convex and rugose but which, in distinction to the first group, form sparse flagellate shoots. These colonies appear within the boundaries of the "sterile zone" and are sometimes very close to the streak of the Actinomycetaceae themselves. /49

The third group includes peculiar smooth colonies or, more often, rugose colonies with entirely even edges, strongly elevated above the agar surface and, depending on the strain of B.mycoides involved, either slimy and glistening or filmy and dull, with no branching whatever. These colonies were encountered only on agar plates to which a filtrate from actinomycetes had been added. Nine cultures of B.mycoides of varying origin were treated in this manner, all of which, on the agar, formed this type of smooth rounded colonies (of the third group).

As noted by Nakhimovskaya, the smooth variants of B.mycoides observed by her have nothing in common with the smooth forms described by Lewis and other authors. The topography of the latter forms is very flat, with a fine granular structure and undulate edges. They never give colonies of the original form on transfer culturing.

The three above groups of colonies are hereditarily unstable modifications, and when transferred to ordinary nutrient agar they produce the normal charac-

teristic colonies of B.mycoides. One of such modified forms, produced under the action of an actinomyces, was transfer-cultured ten times through a medium containing an actinomycetes filtrate. This, however, did not make the smooth form of the B.mycoides colony permanent.

Figure 17 shows photomicrographs of variants of B.mycoides, described by Nakhimovskaya.

From the work of the above authors, the following conclusions can be drawn:

1. B.mycoides has a number of variants differing in colony structure.

2. All variants of B.mycoides may apparently be reduced to the following principal types which most often have a number of subtypes:

a) Rugose form - giving the well-known latticework dextral and sinistral colonies.

b) Smooth form - having more diffuse colonies with wide but relatively short strands, or entirely without strands. In the latter case the edges of the colony resemble the structure of B.anthracoides. /51

c) Forms remotely similar to B.mycoides. The variants described by Nakhimovskaya and other authors could be classified with this group.

3. In nature, the colorless forms of B.mycoides predominate, but forms producing lemon-yellow colonies are sometimes encountered.

4. The forms of B.mycoides mentioned under 2 may be found in nature or produced experimentally from the typical form.

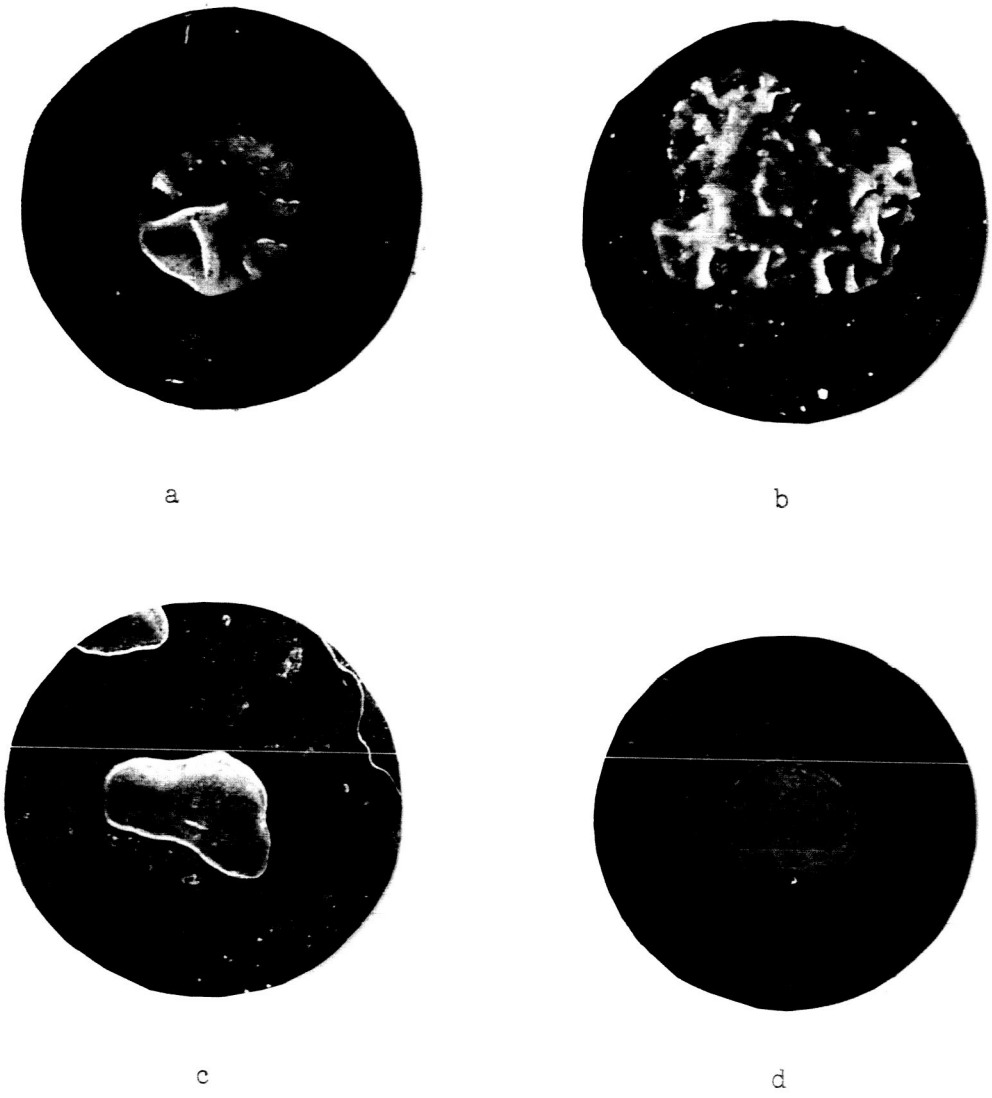
5. Usually, a dissociation in B.mycoides proceeds with relative ease from the rugose form to the smooth. Reversion to the original form is generally difficult.

The experimentally produced forms of B.mycoides include more or less persistent modifications as well as forms with hereditary traits, or mutants.

6. Identification of atypical forms of B.mycoides, when isolated from a natural medium, is extremely difficult and should be further worked out.

7. It is often possible to obtain asporogenic forms of B.mycoides.

8. Cultural and morphological variability often do not run parallel with the variability of biochemical properties. On the other hand, forms differing neither culturally nor morphologically may differ in certain biochemical properties.



a b

c d

Fig.17 Atypical Colonies of B.Mycoides Obtained under the Effect of Metabolic Products of Actinomycetaceae (Photographs a, b, c, and d by Nakhimovskaya)

9. The literature contains some references to isolation of certain variants of B.mycoides in different soils. Up to now, however, there are no reliable data as to the type of soils in which the specific variants of B.mycoides can be found. Nevertheless, some investigations seem to indicate that the smooth forms of B.mycoides correspond to certain soils. For example, Borodulina, in analyzing the gray soils of Fergana, noted the frequent occurrence of varieties of B.mycoides that formed atypical colonies. A similar reference is found in Rautenshteyn's papers on the variability of B.mycoides. He had no difficulty in isolating the smooth forms of B.mycoides from red and gray soils.

In soils from the vicinity of the Yershovskaya Experimental Station (Saratov Oblast), Krasil'nikov, Kriss, and Litvinov (1936) found exclusively smooth variants of B.mycoides. They were unable to isolate the typical forms of this microbe.

In this book, we will give several conclusions as to the distribution of the various subspecies of B.mycoides in the soils of the Soviet Union.

4. Secondary Colonies of B.Mycoides /52

In speaking of variability, we consider it necessary to discuss in some detail the formation, by B.mycoides, of so-called secondary (or daughter) colonies, since these two phenomena are usually closely interlinked.

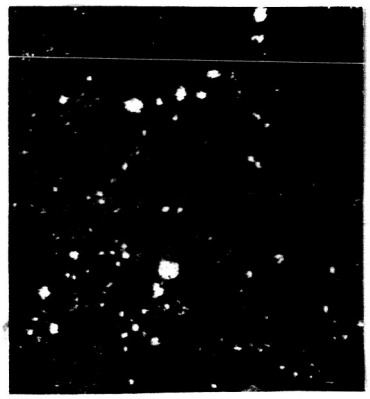


Fig.18 Secondary Colonies of B.Mycoides (according to Lewis)

Bacterial colonies, after a certain period of aging, often give a secondary growth. In the interior of a colony, or on its periphery, small verrucose colonies (daughter colonies) are formed. An extensive literature exists on these formations, and various causes are advanced for their formation. We will give some of the hypotheses.

Secondary colonies may be formed by widely differing bacteria. Preisz (1904) was the first to observe this phenomenon. Various authors have reported the formation of daughter colonies in nonsporulating bacteria. In B.mycoides,

this has been described by Lewis (Fig.18).

Briefly surveying the various concepts on daughter colonies, we will first discuss the work done by Preisz (1904) who asserted that the secondary colonies are formed by germinating spores and several viable cells.

In the opinion of Mellon (1922), the formation of secondary colonies is based on the peculiar stage of the development of a culture that is connected with the phenomenon of pleomorphism. This phenomenon, in turn, depends on the isogamous conjugation of bacteria, which is capable of inducing a modification of their fermentative properties. Thus, the daughter colonies in this case are considered to be formations produced by the altered cells as a result of a sexual process.

Stewart (1926, 1927) had closely related views. Expressing the general hypothesis that growth and its cessation may be explained by an "internal rhythm" including a vegetative phase and a reproductive phase, he concludes that bacterial heredity follows Mendel's law. Homozygotic and heterozygotic forms, with dominant and recessive traits, are said to exist in bacteria. The appearance of daughter colonies is connected with the interconversion of these /53 forms.

In the opinion of Hadley (1922), the daughter colonies are reproduced by gonidia formed asexually by vegetative cells at certain periods of the development. In multiplying, these gonidia yield colonies consisting of cells that are not similar to the mother cells. The size of such cells is close to that of the filtrable individuals. They multiply only feebly and have a low enzymatic capability. From the general viewpoint, it is of course difficult to understand how these delicate cells could successfully multiply on old media.

Enderlein (1925) considers the secondary colonies as a stage of the life cycle of bacteria, including a haploid and a diploid phase. The diploid vegetative cells produce gonidia which, on reversion to the haploid state, are transformed into gametes. They cannot multiply as such, but their copulation does restore the ability to grow, which now proceeds with renewed vigor. This multiplication leads to the formation of daughter colonies.

In contrast to these complicated and speculative hypotheses, Lewis solved the problem of the daughter colonies very elegantly, and at the same time clearly and understandably.

He noted primarily that, among cultures of B.mycoides, there are strong and weak acidifiers. In particular, forms are encountered that ferment saccharose vigorously and other forms that ferment it poorly. On saccharose media, secondary colonies produce only forms that have a low sugar-fermenting action.

Cultures that rapidly ferment carbohydrates will form secondary colonies only on media with bactopeptone or bactotryptophan.

Daughter colonies become extensive about a week after inoculation of the medium. In the beginning, they grow in the older portion of the colony.

It is extremely interesting that cultures prepared from a daughter colony, grown on a saccharose medium, should vigorously ferment saccharose, while the original culture does not possess this property. These new cultures on saccharose agar are no longer able to produce colonies with daughter formations.

Hence, we must assume that secondary colonies arise from individual cells which are capable of assimilating a source of unavailable or scarce carbon to the mass of bacterial cells. Such cells are evidently few in number, since only a few daughter colonies are formed in a given culture. Morphologically, the cells of the daughter colony do not differ from those of the parent culture.

An analogous phenomenon was noted in cultures giving secondary colonies /54 on peptone media. The cultures of B.mycoides obtained from daughter colonies did not produce colonies with daughter formations on the same medium. In this case, the appearance of secondary colonies is connected with the appearance of cells capable of assimilating the decomposition products of peptone. This consideration is confirmed by the fact that filtrates of old media accelerate the formation of daughter colonies.

The physiological differences between various cultures of B.mycoides induced Lewis to stipulate the existence of several groups of this microbe, capable of giving secondary colonies only at a definite composition of the medium for each of these groups.

5. Classification of Variants of B.Mycoides Based on our Observations

The differentiation of types of B.mycoides, as already mentioned, has usually been based on its growth on MPA. It is well known from the literature, however, that the type of B.mycoides colonies is greatly modified depending on the composition of the solid nutrient medium (Holzmüller). We also succeeded in showing that, for example, forms of B.mycoides that differ greatly on MPA give almost identical colonies on potato agar, judging by their macrostructure.

This shows that, by selecting a definite composition for the nutrient medium, it is possible to disclose the structural features of colonies which are masked when the organism is cultured on other media.

Our experience has shown that MPA is by far not the optimum medium for differentiating the colony type in B.mycoides. After various experiments, we found it most convenient to use a medium containing 0.1% Witte peptone and 0.1% K_2HPO_4. This was prepared from tap water and contained 1.5% agar. Media of different compositions, such as MPA, potato agar, etc., permitted confirming the structural features of some type of B.mycoides; however, these specific features are less well delineated and develop more slowly than on peptone agar.

In general, B.mycoides is fairly undemanding as to composition of the nutrient medium, and literature data indicate that this genus can form colonies on agars which contain no additional organic substances. Apparently, the small quantities of water-soluble organic compounds ordinarily present in agar are /55

sufficient to ensure growth of B.mycoides.

However, as shown by our experience, far from all types of agar contain substances available to B.mycoides. For example, on one batch of agar, B.mycoides developed entirely satisfactorily; when the media were prepared from a different agar, no growth of B.mycoides was obtained unless the medium also contained peptone.

Our aim is to develop a medium on which the formation of colonies would be sufficiently rapid, with the structure of the colonies assuming the appearance characteristic for the cultures being tested.

Obviously, on media with a high concentration of nutrients, the formation of colonies is retarded. For this reason, in selecting the percentage of peptone most suitable for our purpose, we first ran a preliminary test. We found that, at various amounts of peptone addition, the individual cultures of B.mycoides retained their characteristic structural features. As soon as the percentage of peptone became too low, the outlines of even the peripheral strands of the colony became effuse. The strands became excessively circinate, which made it difficult to differentiate the type. When the medium contained too much peptone, the colonies became dense and coalescent. The interstices between the strands increase, which prevents a sufficiently accurate analysis of the colony structure of B.mycoides.

After several experiments, we came to the conclusion that a peptone concentration of 0.1% was most suitable for our purposes.

To obtain a certain improvement in the composition of the nutrient medium, as already noted, we also added 0.1% K_2HPO_4. The reaction of the medium was adjusted to pH 7.0 - 7.2.

A study of the collection of our cultures of B.mycoides on this medium permitted the classification of all the multiplicity of forms of this organism into the following principal groups, which might possibly also include some intermediate groups (see Table on p.47).

It should be noted that, in naming the principal groups of B.mycoides, we used previously proposed terminology. The classification into variants is original.

Young colonies of all types of B.mycoides are rather similar, although they differ in the configuration of the bacterial strands. With increasing age, the differences in the various colonies can sometimes be detected by the naked eye.

The traits characteristic for the natural variants of B.mycoides are inherited and persist for a number of years when the cultures are maintained on MPA.

The structural features of colonies of a variant of B.mycoides are often /56 connected with morphological and physiological differences. Thus, in our opinion, the structure of a colony indirectly reflects some of the features of a

culture developed in a natural medium, and does not constitute a derivative phenomenon reproducible only in an artificial environment. It is very likely that we still do not know how to detect most of the features that have been noted, and therefore we must consider the conclusions drawn by various authors, as to the physiological identity of different variants of B.mycoides, to be premature.

Form	Variant	Classification No.
Rugose forms:	a) Ordinary, with random orientation of strands	1a
	b) With annular structure of strands	1b
	c) With strictly oriented strands	1c
Intermediate forms:	a) With elliptically curved strands	2a
	b) With both elliptically curved and radial strands	2b
Smooth forms:	a) With diffuse strands	3a
	b) Forming anthracoidal colonies	3b

Below, we give a short description of the characteristic structural features of colonies of various B.mycoides types, grown on a peptone medium. It should be mentioned again that young colonies of all variants are fairly similar and that the detected differences appear only with time.

Type 1a. Rugose variant of usual type. On visual examination, bacterial strands strictly delineated in width are clearly perceptible. They form a scanty reticular openwork pattern, becoming denser in the central part of the colony. This is a consequence of the thickening of both the principal and secondary bacterial strands. This type of B.mycoides does not show a strict orientation of the strands, whether sinistral or dextral. Usually we note a sinistral rotation (counterclockwise), which is more distinct, in the peripheral part of the colony.

Colonies of this type of B.mycoides show diffuse growth, mainly due to the centrifugal direction of the bacterial filaments in the peripheral part of the colony (Fig.19).

On microscopic examination of the edge of the colony, it is clearly visible that the bacterial strands, most of which are rectilinear, are often curved and that individual filaments may form loopy structures (Figs.20 - 21). /57

At a tender age, the bacterial strand usually shows a less mobile course.

The lateral strands generally leave the main strand at an acute angle, and

no openwork patterns whatever are formed at the point of their separation.

On the periphery of the colony, the internal structure of the bacterial strands consists of slightly curved bacterial filaments. In the old part of the colony, the strands are composed of strongly intertwined and curved filaments. This is evidently the result of the intercalary growth of the filaments with limited opportunity for substantial widening of the strands, owing to the firm attachment of the bacterial cells to the filament.

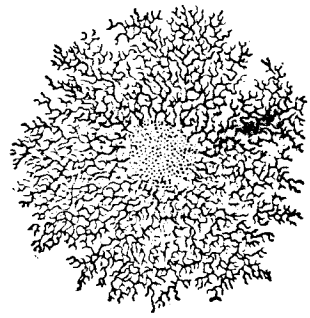


Fig.19 Colony of Ordinary Rugose Form of B.Mycoides on Peptone Agar

An old bacterial strand becomes denser and thicker (Fig.22).

On gradual passage from the periphery to the center, the elements of annular and elliptical structure formed by the strands become more frequent. On the whole, however, an acute-angle system of branching dominates and forms a complex interweaving of strands, forming various geometrical figures.

The structure of the bacterial strands in the peripheral part of the colony is most characteristic for this type of B.mycoides, as for the other types.

Type 1b. Rugose variant with ring structure of the strands. Some cultures of B.mycoides form colonies that greatly resemble the above-described type in all their traits, but a considerable part of both their peripheral and central strands give circinate formations, which may also be accompanied by configurations of elliptical type (Figs.23 - 26). This feature, which is easily established on microscoping the edge of a colony, results in a certain lag of the colony growth on agar relative to that of the rugose forms. In structure, colonies of this type generally resemble the ordinary variant.

On this medium, the cultures of B.mycoides at our disposal gave a sinistral but not particularly pronounced rotation of the strands. /58

Type 1c. Rugose variant with strictly oriented strands. Externally, colonies of B.mycoides of this type are distinguished by the regular orientation

of their strands and by the usually pronounced sinistral rotation. Strands on the edge of the colony are often so strongly curved that their ends curl back to the main portion of the colony.

Fig.20 Peripheral Part of Ordinary Rugose Form of B.Mycoides on Peptone Agar (Photograph)

Fig.21 Peripheral Part of Ordinary Rugose Form of B.Mycoides on Peptone Agar (Sketch)

It is an interesting fact that, whereas an increase in the concentration of agar in the medium (to 2.5%) causes a strong disorientation of the strands in other types of B.mycoides, the habitus of the colony in this type is only slightly modified. Thus, the tendency to sinistral rotation in these variants of B.mycoides is firmly established.

A colony may also have an openwork structure, but the constituent strands are then distinguished by strict orientation and appear as consisting of short straight lines made by a drawing pen.

This strict orientation is also confirmed by the microscopic picture. Fine peripheral strands here proceed in straight lines, with strands and individual filaments running out at acute angles. Elements of convolute type are encountered in small numbers (Figs.27 - 30). /59

Fig.22 Old Strands of Ordinary Rugose Form of B.Mycoides and Peptone Agar (Photograph)

Numerous short shoots usually run laterally outward from the main peripheral strands.

Closer to the center, the principal strands become repand; this is also infrequently observed in the lateral branches. As already noted, we associated this convolution with the intercalary growth of the bacterial filaments composing the strand.

While acquiring a patterned structure, the old strands still maintain a strictly rectilinear orientation.

Type 2a. Variant of intermediate form giving elliptically curved strands. In examining a newly formed colony, a striking feature is the denser confluent structure of colonies of this type of B.mycoides (Fig.31). The colony has a somewhat regressive growth character and lags appreciably in proliferation on

Fig.23 Colony of Rugose Form of B.Mycoides with Annular Structure, on Peptone Agar

Fig.24 Peripheral Part of Colony of Rugose Form of B.Mycoides with Annular Structure, on Peptone Agar (Sketch)

Fig.25 Bacterial Strand from the Older Part of a Colony of the Rugose Form of B.Mycoides with Annular Structure, on Peptone Agar (Sketch)

Fig.26 Peripheral Part of Colony of Rugose Form of B.Mycoides with Annular Structure, on Peptone Agar (Photograph)

Fig.27 Colony of Rugose Form of B.Mycoides with Strictly Oriented Strands, on Peptone Agar

Fig.28 Peripheral Part of Colony of Rugose Form of B.Mycoides with Strictly Oriented Strands, on Peptone Agar (Photograph)

Fig.29 Bacterial Strands from the Older Part of a Colony of Rugose Form of B.Mycoides with Strictly Oriented Strands, on Peptone Agar (Photograph)

Fig.30 Peripheral Part of Colony of Rugose Form of B.Mycoides with Strictly Oriented Strands, on Peptone Agar (Sketch)

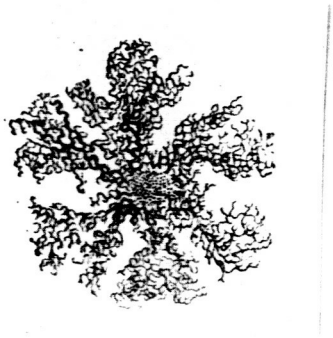


Fig.31 Colony of Intermediate Form of B.Mycoides with Elliptically Curved Strands, on Peptone Agar

Fig.32 Individual Strands of Colony of Intermediate Form of B.Mycoides, on Peptone Agar (Sketch)

Fig.33 Peripheral Part of Colony of Intermediate Form of B.Mycoides, on Peptone Agar (Sketch)

Fig.34 Peripheral Part of Young Colony of Intermediate Form of B.Mycoides, on Peptone Agar (Photograph)

Fig.35 Peripheral Part of Old Colony of Intermediate Form of B.Mycoides, on Peptone Agar (Photograph)

the surface of the agar, relative to colonies of rugose forms of B.mycoides of the same age. It is difficult to determine the orientation of the strands. /64

The above noted depressive growth of colonies of this type is due to the fact that the peripheral strands for the most part are not diffuse and exhibit very steep elliptical convolutions (Figs.32 - 35). There are only very few strands that run in straight lines on the agar.

These oval to circular strands, closer to the center, give a very dense loop pattern.

In the younger part of the colony, the bacterial strands have a plane internal structure, owing to the parallel course of the filaments.

In the older part of a colony, as in the other types of B.mycoides, the strand becomes markedly convolute, but its primary picture remains as before. At the same time, the strand becomes denser and thicker. The cause of this phenomenon, as already noted, is the intercalary growth of the bacterial filaments, which also gives an additional spurious branching.

Type 2b. Variant of intermediate form with both elliptically curved and radial strands in a colony. This type of B.mycoides, in the structure of the colony, apparently combines elements belonging to the preceding form and to the typical rugose form. The principal mass of the colony, even in the peripheral layer, has abundant elliptically and circularly coiled strands. From them, radial shoots frequently proceed outward. The radial strands are surrounded by figured formations, having the character of ellipsoidal loops (Figs.36 - 37). Toward the center, this loop structure becomes increasingly complex.

In many colonies of this type, the structure of the loops is delicate and as a whole has a lacunose character. In this respect, this type of B.mycoides differs greatly from the preceding type, in which the strands as a rule have a coarse structure.

In the central portion of a colony, the strands become denser and coarser, and gradually acquire an internal circinate structure. The strands of a colony usually tend to sinistral rotation. The structure of a macrocolony in general comprises both rugose and ellipsoid types.

Type 3a. Variant of smooth form producing diffuse strands. The colonies of the smooth variants of this microbe differ from all preceding types of B.mycoides in a higher concentration of mucus and broadly diffuse bacterial strands. Proliferation here is not so much in radial direction as in width of the strands. According to some authors, this is due to the weaker adhesion of the bacterial cells to the filaments. In the presence of intercalary growth, this often /66 leads to displacement in the chains, continually forming new parallel filaments. This produces diffusion of the colony, as a result of a certain limitation of its proliferation in width.

Due to this fact, the center of the colony is almost continuously filled with a bacterial mass, and the strands become diffuse (Figs.38 - 41), while the growth of the colony is more restricted.

Fig.36 Peripheral Part of Colony of Intermediate Form of B.Mycoides, with both Elliptically Curved and Radial Strands, on Peptone Agar (Photograph)

Fig.37 Individual Peripheral Strands of Transitional Form of B.Mycoides, on Peptone Agar (Sketch)

The thickened strands of the peculiar configurations running out from the central coalescent part of the colony, usually exhibit sinistral rotation.

A young and not yet completely formed colony of the smooth type of B.mycoides on peptone agar may easily be confused with the rugose type of this microorganism.

Fig.38 Colony of Smooth Form of B.Mycoides, on Peptone Agar

Type 3b. Smooth variant forming anthracoidal colonies. This variant of B.mycoides is very similar to the preceding variant, but the coalescence of the strands is even more complete. For this reason, the edge of the mature colony greatly resembles B.anthracoides, differing only in that some strands, twisted into odd forms, leave the main mass of the colony and penetrate into the agar (Figs.42 - 43). Frequently, in the marginal portion of the colony, a finely capillary structure of the colony is detectable. Sometimes, coalescent slimy colonies of B.mycoides of this type appear completely transparent.

In young colonies, there occasionally is a distinct orientation (usually sinistral) of the strands on the agar surface. During this period, a colony of this type greatly resembles the rugose variant.

Below, we will use the above serial numbers of the variants of B.mycoides as subscripts in our definitions.

In speaking of the typing of B.mycoides colonies from their growth on nutrient media, including 0.1% peptone agar, we must again emphasize that this differentiation is possible only when studying the growth dynamics of the colony. As noted before, young colony, even of a smooth variant of B.mycoides, is almost identical with a colony of rugose type. It is true that the peripheral strands of the former have a more convolute shape, but only an investigator who has /68 done much work on B.mycoides can make a correct typing of a young colony. The same difficulty is encountered in studies on the intermediate forms which, even in young colonies, tend to show a more convolute pattern.

Only after several days in the incubator will the picture, characteristic of a definite type of B.mycoides, be distinctly outlined. Similarly, old

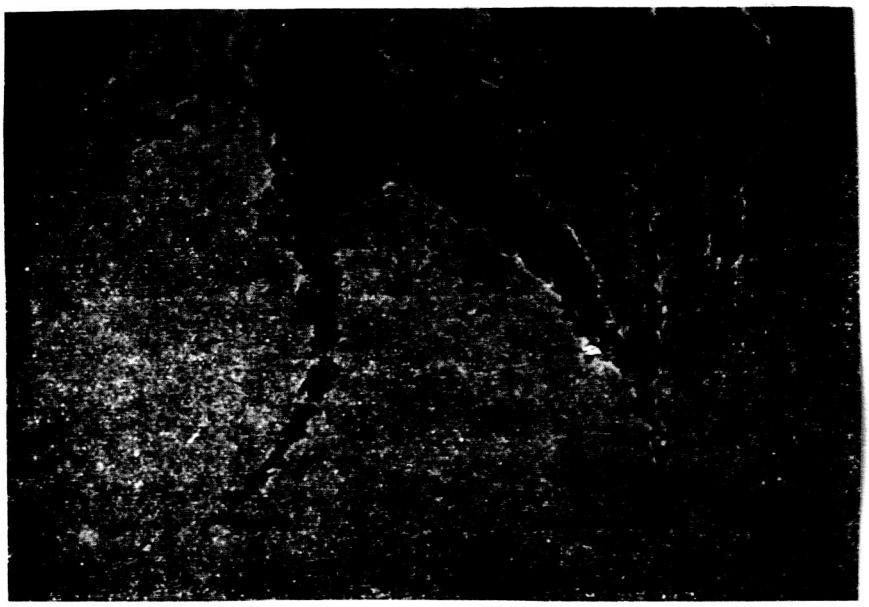


Fig.39 Peripheral Strands of Young Colony of Smooth Form of B.Mycoides, on Peptone Agar (Photograph)

Fig.40 Peripheral Part of Formed Colony of Smooth Form of B.Mycoides, on Peptone Agar (Sketch)

Fig.41 Individual Diffuse Strand of Old Colony of Smooth Form of B.Mycoides, on Peptone Agar (Sketch)

colonies of B.mycoides are unsuitable for typing. In such colonies, even the strands of peripheral type become thicker in time, acquiring a repand structure and losing their characteristic topography. The analyst will encounter some elements characterizing the type, but they will more or less be "retouched".

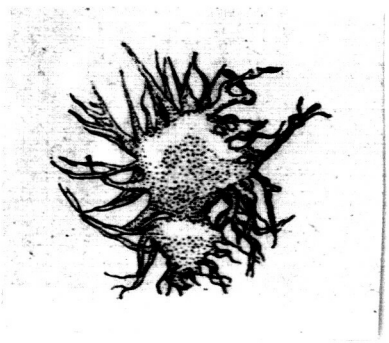


Fig.42 Colony of Anthracoidal Form of B.Mycoides, on Peptone Agar (Sketch)

Fig.43 Peripheral Part of Colony of Anthracoidal Form of B.Mycoides, on Peptone Agar (Sketch)

It is of special interest that not only the composition of the medium, but also the agar concentration, have a great influence on the structure and orientation of the colony.

For example, on increasing the percentage of agar from the usual level of 1.5% to 2.5%, the rugose variants of B.mycoides (not always, but in some cases) acquire the colony structure characteristic of the intermediate forms, with

restricted proliferation of the colony on the surface of the medium. In other words, the strands here are more convolute. Table 5 gives data from an experiment with 16 cultures of B.mycoides. As shown, in five cases a transition /69 from the rugose to the intermediate type occurred. This transformation was reversible, and culturing the modified colony on a standard medium restored its former properties.

TABLE 5

INFLUENCE OF AGAR CONCENTRATION ON THE TYPE OF COLONY FORMED

Culture		Type of Colony, on Peptone Medium with Agar Content of (in %)		
		1.5	2.0	2.5
Igarka	1	1a	1a	2a
	2	1a	1a	1a
	3	1a	1a	1a—2b
	4	1a	1a	1a
	5	1a	1a	1a—2b
	6	1a	1a—1b	1a—1b
Moscow				
	1	1a	1a	1a
	2	2b	2b	2b
	3	1a	1a	1a
	4	1a	1a	2b
	5	1a	1a	1a—2b
Central Asia (Pakhta-Aral)				
	1	3a	3a	3a
	2	1a	1a	2b
	3	1a	1a	1a
Samarkand				
	4	1a	1a	2a—2b

Note: If two types are given, colonies of intermediate type were formed. For example, 1a - 2b means that the colony formed had a character intermediate between the ordinary rugose type and the plumose-ellipsoidal type.

It is of considerable interest that an increase in agar content of the medium generally disorients the rotation of the strand in the rugose forms (Type 1a and 1b) and the intermediate forms. On media with 2.5% agar, the sinistral forms at our disposal assumed a random or sometimes a dextral orientation. Only Type 1b and the smooth forms maintained their usual sinistral rotation as well as their characteristic colony structure on the concentrated media.

This makes it obvious that the state of the water film on the agar surface is of great importance in typing the colony structure. For this reason, such processes as the drying of agar poured into Petri dishes may have an extensive /70 influence on the habitus of a B.mycoides colony. According to our observations, even the temperature of pouring the agar may have an influence. All these effects do not eliminate the specific differences of colonies of various B.mycoides

forms, but make typing difficult.

Therefore, we always observed strict rules in our work. The agar was poured only after extensive cooling, and the dishes with the nutrient medium were never allowed to dry out. For the control, we always transferred well-studied cultures of definite types. The cultures of B.mycoides whose type had been established were transferred not less than four times to a fresh medium of the same composition.

In discussing our above classification of B.mycoides, the question as to the stability of the characteristic structural traits of a given type of colony, in cultures on other media, may arise.

There is an entirely definite answer to this question: The distinctive features of the colony type persist on other nutrient media, but to a more or less pronounced degree. Below, we give a brief description of colonies of our forms of B.mycoides, on potato agar and meat-peptone agar*.

Touching only on the main characterization factors of the various colony types of B.mycoides when grown on these media, the following statements can be made.

Variant 1a. Gives rather dense colonies on the MPA surface. These colonies show a considerable increase in the main-strand interspaces. Therefore, the central part of the colony has almost no areas that do not contain a bacterial scum.

The peripheral strands in a given colony are rather long and, under the microscope, can be seen to consist of generally rectilinear filaments and with lateral branching. Both main and secondary strands are distinguished by a strictly defined topography. At the sites of branching there is almost no open-work pattern left.

Closer to the center, the strands become broader but, like the secondary branching-off strands, still preserve their rectilinear direction. The lacunae formed by the interwoven strands almost never are round but have a polygonal structure.

The circinate structure inside the bacterial strand appears only in the old portion of the colony (Figs.44 - 47).

On potato agar, the colonies are more diffuse and lacunose. Here, the proliferation of the bacterial mass in the space occupied by the colony is not as dense. The strands usually have a sinistral orientation. The curvature /72 here is more pronounced than on peptone or meat-peptone agar (Fig.48).

The microscopic structure of the colony is generally quite similar to that formed on the surface of MPA.

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* Dry Nevizhin's medium was used in preparing the MPA.

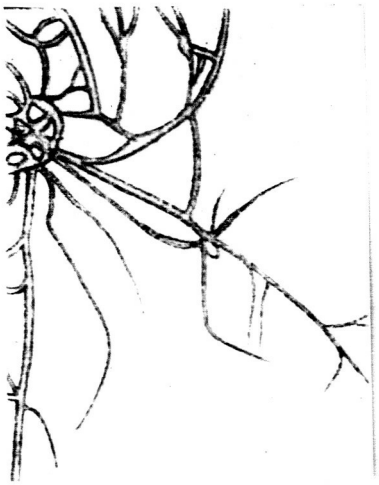

Fig.44 Edge of One-Day Colony of Rugose Form of B.Mycoides, on MPA (Sketch)

Fig.45 Edge of Three-Day Colony of Rugose Form of B.Mycoides, on MPA (Sketch)

Fig.46 Edge of Five-Day Colony of Rugose Form of B.Mycoides, on MPA (Sketch)

Fig.47 Peripheral Strand of Old Colony of Rugose Form of B.Mycoides, on MPA (Sketch)

Variant 1b. On MPA, gives colonies outwardly similar to the preceding, but differing somewhat in the microscopic structure of the strands. Here, the peripheral non-branching portion is rather short. There are numerous annular and oval configurations, sometimes with radial divisions.

Somewhat closer to the center, the strands broaden greatly. The expanded strand gradually acquires a sinuous and, occasionally, a beaded structure.

Fig.48 Peripheral Portion of Colony of Rugose Form of B.Mycoides, on Potato Agar (Photograph)

Thus, a tendency of this type to the formation of coils of annular configuration is noted on MPA. /73

On potato agar, this feature is detected rarely, and then only at a certain age of the colony. In young colonies, the structure cannot be distinguished from that of the usual rugose type just as, in the old colonies, the features of the structure are poorly identified.

Variant 1c. On MPA, forms colonies which show an openwork pattern in the peripheral layer of the structure. The main strands in this type are thinner than in the usual rugose type. They have numerous shoots, running out at acute angles.

A thickened strand in the older portion of the colony is divided like a

tattered rag from the mass of shoots running out from it. The sinuosity usually present in the structure of an old strand is also noted here. However, the aged strand in this case does not thicken as much as in the usual forms of B.mycoides, so that the colony appears more delicate.

Fig.49 Peripheral Portion of Colony of Rugose Form of B.Mycoides with Strictly Oriented Strands, on Potato Agar (Photograph)

On the whole, however, the outward appearance of such colonies greatly resembles the ordinary forms of B.mycoides, and it is difficult to detect them on MPA without analysis on peptone agar.

The same general openwork character and stricter orientation of the main /74 strands are also manifested by this type, in colonies grown on potato agar (Fig.49).

In general, on MPA and potato agar, colonies of Type 1c are more difficult to distinguish than on peptone agar.

Variant 2a. On MPA, forms colonies whose outward appearance is entirely similar to colonies of the ordinary rugose forms. However, under the microscope, structured bacterial strands are noted in the peripheral layer from which shoots penetrate into the medium in a centrifugal direction. The curved strands take on an annular to ellipsoidal form. In young colonies, this curvature is not characteristic of the peripheral layer and is noted only closer to the center.

In a given colony, closer to the center, the strands broaden and assume a very coarse outline; but the curved pattern remains fully perceptible. Internal sinuosity of the strand is noted in the oldest portion of the culture.

Thus, the tendency of some forms of B.mycoides to give elliptically-rounded strands is also manifest on MPA. Here, this property is less clearly defined than on a peptone medium, since the growth of the colonies is more diffuse.

A still less differentiated type of colony is obtained on potato agar, where growth is more diffuse. The structure of the colony differs from the typical rugose form only by the presence of abundant annular structures in the peripheral strands and by the less agitated course of the linear spreading filaments.

Fig.50 Peripheral Part of Colony of the Intermediate Form of B.Mycoides, on Potato Agar (Photograph)

In the older portions of the colony, alongside the ordinary reticulate structure, there frequently are rounded-ellipsoidal formations, not characteristic of the rugose forms of B.mycoides (Fig.50).

Variant 2c. On MPA and on potato agar, forms colonies not distinguishable from the preceding type of B.mycoides but probably still closer to the colonies of the rugose type.

These media give no clear-cut picture and can be used only with difficulty for typing the intermediate forms of B.mycoides.

Variant 3a. At a young age on MPA, its colonies differ little in appearance from the colonies of the rugose forms. Later, however, the bacterial mass fills the space between the strands not only at the central part of the colony but also in its peripheral layer. This gives the surface of the colony a /75 smoothed topography.

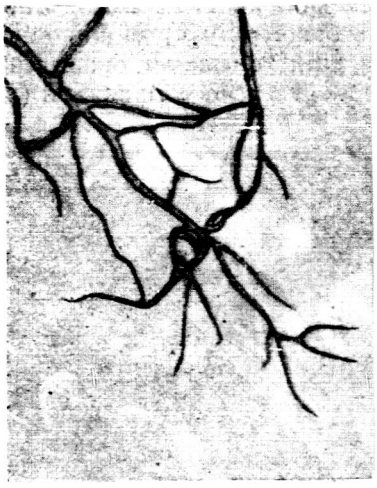

Fig.51 Edge of One-Day Colony of Smooth Form of B.Mycoides, on MPA (Sketch)

Fig.52 Edge of Three-Day Colony of Smooth Form of B.Mycoides, on MPA (Sketch)

Fig.53 Edge of Five-Day Colony of Smooth Form of B.Mycoides, on MPA (Sketch)

Fig.54 Edge of Seven-Day Colony of Smooth Form of B.Mycoides, on MPA (Sketch)

Fig.55 Edge of Seven-Day Colony of Smooth Form of B.Mycoides, on MPA (Sketch)

Fig.56 Structure of Colony of Smooth Form of B.Mycoides, on Potato Agar (Photograph)

Fig.57 Edge of Three-Day Colony of Anthracoid Form of B.Mycoides, on MPA (Sketch)

Fig.58 Edge of Five-Day Anthracoid Form of B.Mycoides, on MPA (Sketch)

The strand of the peripheral part of a young colony has numerous coils, which do not interfere with the predominantly linear progress of growth. The aging strand thickens and becomes surrounded by massive zigzag beads (see Figs.51 - 55).

When a colony ages, the curved elements in the peripheral part increase. The strands become patterned coils. They give off numerous lateral shoots, imparting a villous appearance to the strands.

In the central portion of the colonies, the strands broaden excessively and begin to coalesce, leaving only in some places lacunae not yet overgrown. The microscope distinctly shows that these wide strands consist of circinate fibers. The darker contours still mark the earlier positions of the main strands.

Still persisting isolated secondary and tertiary strands also show a contoured structure.

The confluent area gradually spreads to the peripheral layer of the colony. The edge of the colony recalls B.mycoides, but within the medium there are numerous fine strands with figured, nodular formations.

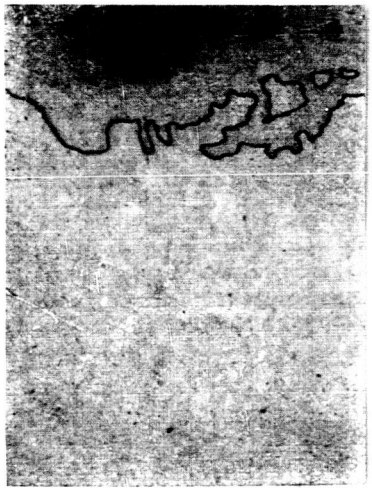

Fig.59 Edge of Seven-Day Colony of Anthracoid Form of B.Mycoides, on MPA (Sketch)

On potato agar, the development of the smooth colony is similar to that /78 just described. The growth, however, is more diffuse, and the broadening of the main strands more restricted. This makes the outward appearance of colonies of the smooth variants similar to that of the typical form. However, the internal structural elements remain specific (Fig.56).

Thus, on the edge of a smooth colony, the bacterial strands are highly repand, running almost perpendicular to the main direction of the strands. In some smooth variants of B.mycoides, the curved filaments are so close together

that a cursory glance would take them for straight lines.

The peripheral strands of the smooth forms of B.mycoides have more side branches than those of the rugose forms.

In the older portions of the colony, the strands are considerably diffuse and become undulate. Their projections often exhibit an intricate pattern. The secondary strands, interconnecting the main ones, have a similar structure.

In an aged colony, the strands are diffuse both at the periphery and at the center, but their outlines are fully retained so that there is no continuous growth filling the interspaces.

Soft, complex-figured, but blurred contours are characteristic of the smooth forms of B.mycoides when grown on potato agar.

Thus, the tendency to form more diffuse colonies is characteristic of the smooth forms of B.mycoides when grown on meat-peptone and potato agars.

In the variant 3b, the colonies in the young state on MPA show no substantial difference from the preceding type. They are characterized by the same figured structure of the young and aged strands. The latter, however, have a still greater tendency to diffusion, so that there are almost no individual strands in the final colony. The edge of the colony over almost the entire length has a trichomous-undulate contour greatly resembling a colony of /79
B.anthracoides. Only in some places do figured shoots extend into the depth of the medium from the colony (Figs.57 - 59).

It is quite impossible to guess at the position of the main strands in the depth of the colony.

On potato agar, a young colony of the anthracoid variant of B.mycoides has strands rich in variously patterned figures, characteristic for the colonies of the smooth forms. On aging of the culture, both the peripheral and the main strands proliferate strongly but give no continuous growth on potato agar. Along the edge of the colony, the broad strands consist of broken bacterial filaments running in random directions. Their edges show numerous shoots.

In the central part of the colony, the strands assume a slimy consistency.

Because of the absence of growth into the strand interspaces on potato agar, the anthracoid colonies exhibit the habitus typical for B.mycoides.

This is also emphasized by the diffuse growth of colonies on potato agar.

This description of the different variants of B.mycoides on three media justifies the conclusion that the distinctive traits characteristic of certain cultures are constant and firmly established.

At the same time, these distinctive traits are prominent on some media such as peptone agar, while on other media such as potato agar, they recede into the background. This makes it entirely logical that we specified peptone agar, with 0.1% peptone, as most suitable for typing B.mycoides colonies.

Experiments with a larger collection of cultures of B.mycoides on a new medium will permit a classification of the multitudinous cultures of this microorganism into several main groups of identical macrocolony structure.

It might well be that further work on this problem will require some refinement of the proposed classification.

CHAPTER III /80

MORPHOLOGICAL, PHYSIOLOGICAL, AND CULTURAL TRAITS OF B.MYCOIDES

1. Microscopic Picture

In this Chapter, going more deeply into the problem, we will describe the distinctive traits of a culture of B.mycoides, with respect to its morphology and physiology. Wherever possible, we will indicate the features of the variants of this microorganism. Let us begin the analysis of the material with the structure of the cell and its forms in B.mycoides.

According to the description by Lehmann and Neumann, B.mycoides has large rods, slightly rounded at the ends, 1.6 - 3.6 μ long and 0.8 μ wide. The cells are often connected in chains, resembling the filaments of the anthrax bacillus.

Other authors give slightly different dimensions for the B.mycoides cell. These data are compiled in Table 6, from which the figures presented for the atypical or degenerate cells have been omitted deliberately.

In analyzing these figures, it is easy to conclude that the thickness of the cells in various cultures of B.mycoides often differs substantially. The same conclusion can be drawn from the photomicrographs by Lewis (1932), Oesterle (1930) and other authors.

No author, except Kushnarev, has ever observed a cell thinner than 0.6-0.8 μ for B.mycoides.

Work of basic interest, devoted to the dimensions of the B.mycoides cell, was published by Novogrudskiy and Kononenko (1935). They attempted to find, in the cell size, a trait analogous to the genotypic traits of a higher organism. Novogrudskiy seeks a precondition for this in the work of Uspenskiy, who showed that in the cells of spirogyra, the mass of the nucleus increased or decreased by an integral number of times. This transformation leads to the transformation of one ecotype of the spirogyra into another.

Novogrudskiy considers that the length of the cell is not characteristic /81 for B.mycoides and depends on the culture conditions of the medium. The cell diameter, however, varies very little during the entire lifetime of a given culture. Exceptions occur if phenomena of lysis and nitrogen starvation are present. Thus, the diameter of the cell may be considered a trait that is only slightly dependent on the external conditions and is more constant than even the growth temperature of a culture.

Having isolated a considerable number of cultures of B.mycoides from various soils, Novogrudskiy studied the cell diameter with the object of finding the principal ecological types of this microorganism, under the conditions of its natural habitat. He selected only typical so-called "rugose" forms of B.myc-

oides for his studies.

The cultures were grown on an agar medium containing 1% peptone, 0.5% K_2HPO_4, and 2% agar-agar. The preparations were stained with Manson's stain.

TABLE 6

DIMENSIONS OF CELLS OF B.MYCOIDES ACCORDING TO VARIOUS AUTHORS (IN μ)

Investigator	*Culture of Bacteria*	*Width of Cell*	*Length of Cell*
Eisenberg	*Bac, mycoides*	0.9	1.6—2.4
Marshall Ward	" "	1.75	3.0—6.0
Varlikh	" "	1.1—1.15	3.4—6.0
Migula	" "	0.94	1.6—2.4
Holzmüller	*Bac. mycoides* Type β	1.2—1.8	3.47—4.92
	" " " γ	1.2—2.65	2.31—4.62
	" " " δ	0.87—1.39	2.08—4.17
Perlberger	*Bac. mycoides* Holzmüller δ	0.76	2.24—3.5
Perlberger	*Bac. mycoides* Holzmüller γ	1.02	4.59—5.52
Perlberger	*Bac. mycoides* Flügge № 3	1.2	3.06—4.6
Perlberger	*Bac. mycoides* Flügg № 4	1.22	3.06—4.5
Perlberger	*Bac. mycoides* Gersbach	1.53	2.82—6.0
Lehmann and Neumann	*Bac. mycoides* Flügge	0.8	1.6—3.6
Bergey	Same	0.8—1.0	2.0—4.0
Kushnarev	Various cultures of *Bac. mycoides*	0.2—0.5	3—6
Novogrudskiy	" "	0.8—1.47	—
Stapp and Zycha	" "	1.5	5.0
Oesterle and Stahl	" "	0.5—0.6	0.5—1.5
Rautenshteyn	*Bac. mycoides*—Northern cultures	1.1—1.2	3.6—6.0
	Bac. mycoides—Southern cultures	1.4—1.6	3.0—5.0

The measurements were made with an ocular screw micrometer. /82

This work permitted the following conclusions:

1. The diameter of a B.mycoides cell is rather constant in the different cultures and represents a characteristic value varying between 0.84 and 1.47 μ.

2. No gradual transition from the thin to the thick form was observed in this material. On the contrary, there are certain dimensions of the cell diameter of B.mycoides which are characteristic of entire groups of cultures. /83 The cell diameter of some groups does not change gradually into values characteristic of other groups; rather, there are some discontinuities. Figure 60 gives an idea of the thickness of cells of various types of B.mycoides.

The following are the principal types:

a) thin - a, with a cell diameter of 0.84 - 0.91 μ;
b) medium - b, with a cell diameter of 1.05 - 1.12 μ;
c) thick - c, with a cell diameter of 1.40 - 1.47 μ.

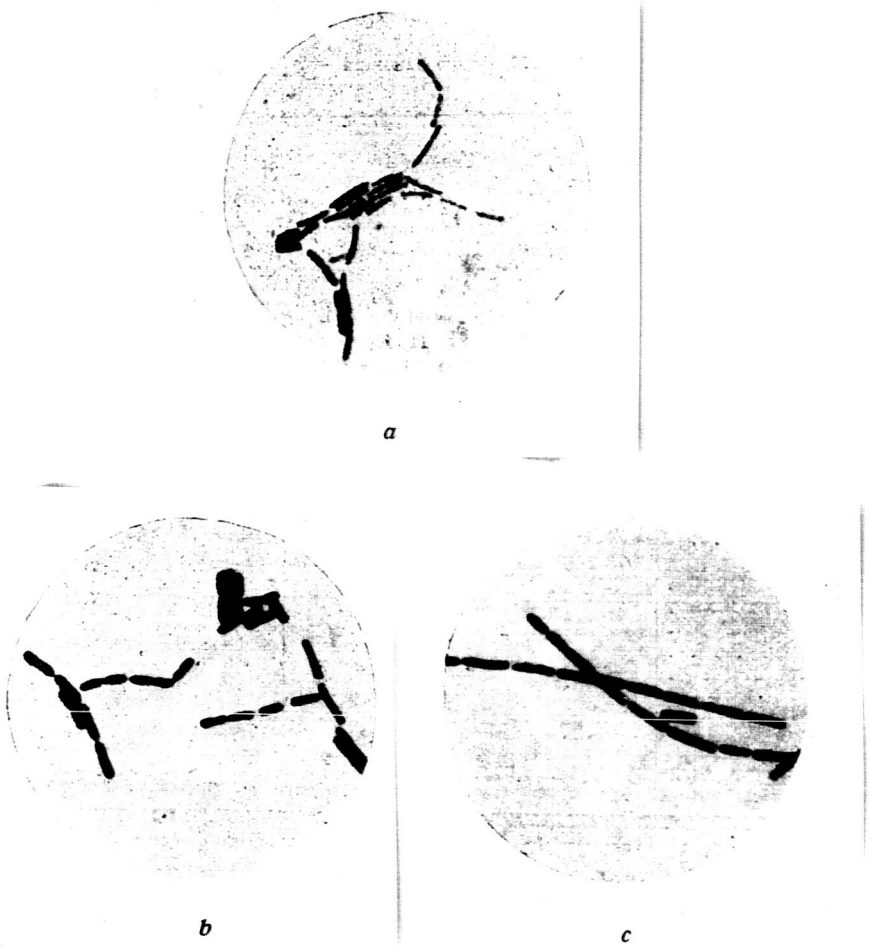

Fig.60 Thickness of Cell in Various Cultures (according to Novogrudskiy):
a - B.mycoides with thin cells; b - B.mycoides with cells of moderate thickness; c - B.mycoides with thick cells

3. Certain numerical relations exist between the cell diameters of these principal forms of B.mycoides. The cell diameter of each succeeding group is the $\sqrt[3]{2}$ = 1.26 times as great as the diameter of the preceding group.

4. The medium-thickness forms of B.mycoides are most widely distributed in the soils. The thin and thick variants are only infrequently encountered. Of 28 cultures studied, three were thin, 17 were medium, and eight were thick. In various soils, the following distribution of B.mycoides types were found:

Number of forms:	Medium	Thick	Thin
Isolated from podzols	8	3	1
From chernozems	3	-	2
From red earths	1	5	2
From other soils	5	-	-

The medium forms showed the least fluctuation in diameter among the individual cells of the same culture. For this reason, the variation curves here were most regular. This could be noted even on direct microscopic analysis. Cells of varying thickness were found in the filaments of the thick and thin cultures. Such phenomena were not observed in the intermediate cultures. In the cultures of some thick rods, "super-thick" forms were found with a granular cell content and a diameter of 2.1 - 2.17 μ. This diameter equals that of the thick rods, multiplied by $\sqrt[3]{2}$.

In some cases there were swollen, subrounded large cells recalling the "gonidangia" of Oesterle (Fig.61).

Fig.61 Greatly Thickened Cell of B.Mycoides (according to Novogrudskiy)

In cultures of the thick rods the transition to the "super-thick" cells was gradual. Therefore, in this particular case, the author himself considered his formula arbitrary (Fig.62).

The thick, medium, and thin forms differed in cultural and biochemical properties, as we will demonstrate below.

Under laboratory conditions, some cultures lost their peculiar properties. These changes were noted only in the thin and thick forms, but not in the /84 medium forms. Such changes began to be noted 2 - 3 months after isolation of the cultures from the soil.

Within six months, all the thin forms had turned into medium forms. In exactly the same way, most of the thick forms also became medium forms.

Thus, the thin and thick forms proved to be unstable under laboratory conditions. Hence we may conclude that, despite the very great interest of the above paper, the attempt to use the diameter of the B.mycoides cell as a primary distinctive trait, characterizing the culture, ended in failure. This trait proved less stable than the relation of the bacteria to the temperature, to sources of carbon, nitrogen nutrition, etc.

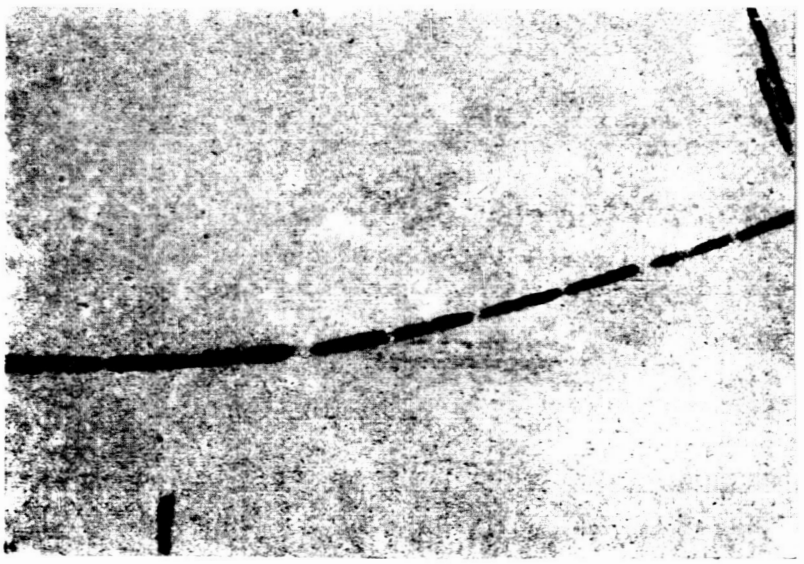

Fig.62 Gradual Thickening of Cells in a Filament of B.Mycoides (according to Novogrudskiy)

This was largely to be expected on the basis of earlier work by Buchner, who showed that the composition of the nutrient medium has a great influence on the cell diameter.

Nevertheless, the fact of the existence of cells of different form in the individual cultures of B.mycoides seems reliably established. In particular, this is confirmed by micrographs of our cultures taken under the electron microscope at the Institute of Microbiology, USSR Academy of Sciences, by A.Ye.Kriss and Ye.A.Rukina (Figs.63 - 64).

We do not believe in the reliability of the mathematical relations between the width of the cells of various cultures of B.mycoides. Novogrudskiy and Kononenko used stained preparations, thus distorting the true size of the cells which were possibly not proportionally equal in cells of different thickness.

Since the cell diameter of the individual variants of B.mycoides described by us might not be identical and not characteristic for a given group of cultures, we decided to make a special experiment to refine the technique.

Fig.63 Cells of Smooth Variants of B.Mycoides, under the Electron Microscope (Photograph by Kriss and Rukina, Magnification × 22,000)

Fig.64 Cells of Rugose Variants of B.Mycoides, under the Electron Microscope (Photograph by Kriss and Rukina, Magnification × 22,000)

TABLE 7

VARIATION OF WIDTH OF CELLS OF B.MYCOIDES ON STAINING (CULTURES GROWN ON PEPTONE AGAR)

Cultures Studied	Character of Preparation	Frequency of Encountered Cell Widths, Expressed in Micrometer Divisions											Average Width	Change on Staining	Shrinkage of Cell on Staining (in %)	Width of Cell (in μ)	
		13	14	15	16	17	18	19	20	21	22	23				Before Staining	After Staining
Igarka, rugose No. 10	Stained	1	11	18	13	4	--	--	--	--	--	--	15.2	2.9	16.0	1.45	1.22
	Unstained	--	--	--	1	11	18	10	8	2	1	--	18.1				
Same, No. 4	Stained	2	9	7	13	17	2	--	--	--	--	--	15.8	2.8	15.6	1.49	1.26
	Unstained	--	--	--	4	9	13	7	9	6	--	--	18.6				
Frunze, transitional No. 3	Stained	--	1	4	6	19	15	2	--	--	--	--	17.0	3.6	17.4	1.65	1.36
	Unstained	--	--	--	--	2	4	5	12	13	11	3	20.6				
Same, No. 11	Stained	--	3	4	15	18	8	1	1	--	--	--	16.6	4.4	20.9	1.68	1.33
	Unstained	--	--	--	--	--	6	6	8	11	15	5	21.0				
Golodno Step', smooth	Stained	--	--	3	20	18	19	4	1	--	--	--	17.1	5.0	22.7	1.77	1.37
	Unstained	--	--	--	--	--	--	1	4	6	18	20	22.1				

In order to check the effect of Manson's Methylene-Blue stain on the /85 size of the cell, we conducted a number of check tests whose results are given in Table 7. We made parallel measurements by an ocular screw micrometer on stained and unstained preparations of the same culture of B.mycoides. To the unstained preparation a small amount of agar was added, so as to fix the cells in their position, and a small amount of Methylene Blue to render the outline of the cell contours better visible. In this experiment, we studied five cultures taken from different soils. The cultures were grown on the peptone agar recommended by us for the typing of B.mycoides*. Before beginning the work, all of the cultures were purified by repeated transfer culturing from the edge of the colony. Usually we measured 40 - 50 cells in one preparation. In Table 7 and all subsequent Tables, we indicate the frequency of occurrence of cells of specified size, expressed in readings of the micrometer. In addition we give the mean values expressed in scale divisions of the micrometer screw, in microns. In work with an oil-immersion system (× 120), and an × 10 ocular, one turn of the micrometer screw was equal to 0.08 μ.

It must finally be noted that we used an 18-hour culture for measurement. /88

As indicated in the Table, not all cultures of B.mycoides have the same size. For example, the Igarka cultures had the smallest cells, the cells of the cultures isolated from Frunze soils were somewhat larger, and the cells of cultures from the Golodno Step' (near Tashkent) were still larger. However, the ratio of the values does not fit into the rule established by Novogrudskiy. It is true, Novogrudskiy repeatedly emphasized that, under laboratory conditions, the cell size of the most widely differing types of B.mycoides came to a common level. In our work, we had to use cultures of B.mycoides that had been kept for a relatively long time under laboratory conditions.

It is obvious from Table 7 that the intermediate and smooth forms had larger cells. Below, we will discuss the extent to which this rule can be treated as a general law.

The staining of the cells led to a considerable shrinkage in cell diameter, but not to the same extent in all cultures. The large cells shrank more than the thin ones. Hence, it is easy to draw conclusions as to the value of any mathematical rules based on a study of stained preparations.

Data similar to those in Table 7 are also given in Table 8. For these measurements, we took various cultures of B.mycoides, 18 hours old, but using this time MPA as medium. The conditions were otherwise the same as those described above.

The experimental results show that young cells of various subspecies of B.mycoides do not differ much when grown on MPA. This proposition was checked and confirmed on the more extensive data discussed below. Here again, after staining, the B.mycoides cells became appreciably smaller in diameter.

We then took up the question how age differences affected the cell width

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* The formula for the medium was given above.

TABLE 8

VARIATION IN WIDTH OF CELLS OF B.MYCOIDES ON STAINING
(Cultures Grown on MPA)

Culture	Character of Preparation	Frequency of Encountered Cell Widths, Expressed in Micrometer Divisions											Average Width	Change Due to Staining	Shrinkage of cell on Staining	Width of Cell (in μ)	
		12	13	14	15	16	17	18	19	20	21	22				Before Staining	After Staining
Moscow, rugose No. 15	Stained	1	8	15	22	3	—	—	—	—	—	—	14.4	4.0	21.7	1.47	1.15
	Unstained	—	—	—	—	—	15	25	8	6	5	1	18.4				
Frunze, intermediate No. 3	Stained	—	3	9	19	7	4	—	—	—	—	—	14.0	3.3	19.1	1.38	1.12
	Unstained	—	—	—	—	4	24	15	3	—	—	—	17.3				
Same, No. 4	Stained	—	9	15	13	3	1	—	—	—	—	—	14.2	2.7	15.9	1.36	1.14
	Unstained	—	—	—	2	20	16	9	5	1	1	—	17.0				
Same, No. 15	Stained	3	14	20	13	—	—	—	—	—	—	—	13.8	3.2	18.8	1.36	1.10
	Unstained	—	—	—	3	14	15	10	3	2	—	—	17.0				
Same, No. 16	Stained	1	6	25	24	2	2	—	—	—	—	—	14.4	4.5	23.8	1.51	1.15
	Unstained	—	—	—	—	—	—	18	16	6	3	—	18.9				
Samarkand, intermediate No. 1	Stained	2	11	19	14	2	—	—	—	—	—	—	14.1	3.2	18.5	1.38	1.13
	Unstained	—	—	—	4	9	22	17	5	—	—	—	17.3				
Same, No. 2	Stained	—	14	24	11	1	1	—	—	—	—	—	14.0	3.3	18.4	1.38	1.12
	Unstained	—	—	—	1	10	21	17	3	2	—	—	17.3				
Sumy, rugose No. 3	Stained	—	11	21	14	1	1	—	—	—	—	—	14.2	2.6	15.5	1.34	1.14
	Unstained	—	—	—	5	14	15	13	1	—	—	—	16.8				
Sumy, intermediate No. 4	Stained	2	12	22	15	1	—	—	—	—	—	—	14.0	3.3	18.4	1.38	1.12
	Unstained	—	—	—	—	7	19	19	2	—	—	—	17.3				
Golodno Step' smooth No. 10	Stained	1	7	10	18	4	1	—	—	—	—	—	14.0	3.7	20.2	1.46	1.17
	Unstained	—	—	—	—	—	1	28	13	15	9	2	18.3				

in the different forms of B.mycoides. To answer this question we measured live cells of several cultures of B.mycoides grown on peptone agar and meat-peptone agar. For several days, we measured cells taken from the edge of the colonies and older cells from the center. Table 9 gives the analytical data. The following conclusions must be drawn:

1. With age, on all media, the cell diameter increases in both the rugose and smooth forms of B.mycoides. We associate this increase in size during the

TABLE 9

VARIATION IN CELL WIDTH OF B.MYCOIDES DURING ONTOGENESIS

Site of Isolation of Culture	*Type of Variant*	*Average Width of Cells of Various Age (in μ)*		
		6 *hours*	1 *day*	3 *days*
	I. Culture on Peptone Agar			
Near Igarka	Rugose	1.42	1.48	1.65
" Moscow	"	1.41	1.42	1.58
" Sumy	Intermediate	1.66	1.69	1.85
" Frunze	"	1.57	1.74	1.74
" Samarkand	"	1.63	1.79	1.87
Pakhta-Aral (Golodno Step')	Smooth	1.68	17.50	2.10
Same	"	1.68	1.83	2.05
"	"	1.90	1.96	2.24
	II. Culture on MPA			
Near Moscow	Rugose	1.47	1.48	1.64
Pakhta-Aral	Smooth	1.46	1.46	1.98
	III. Average Cell Dimensions of Different Variants (on peptone agar)			
	Rugose	1.41	1.45	1.66
	Intermediate	1.62	1.74	1.82
	Smooth	1.79	1.85	2.13
	IV. Ratio of Cell Sizes of Various Age (on Peptone Agar)			
	Intermediate / Rugose	1.15	1.20	1.10
	Smooth / Intermediate	1.10	1.07	1.17

process of ontogenesis with the process of aging of the cell, and the subsequent spore formation. As shown in the corresponding control experiments, the swelling of the cell begins to show before formation of the prosporangium and thus is not a process of purely mechanical tension of the cell by the blastospore being formed. In general, cells with fully formed spores were omitted from the measurements. /90

2. On aging, the smooth forms of B.mycoides give thicker cells than the rugose forms. The transitional variants occupy an intermediate position.

3. On peptone agar, the young cells of the smooth variants of B.mycoides are thicker than those of the rugose forms. On MPA, the size of the young cells of all variants of B.mycoides is identical. Both on MPA and peptone agar, aged cells thicken and reach the dimensions characteristic for a certain variant of B.mycoides. The American author Lewis had the impression that cells of B.mycoides thicken during aging.

4. On a peptone-poor medium, for all ages of cells, the ratio of the width of the thicker variant to that of the preceding variant was close to 1.10 - 1.15. On MPA, this difference can be attributed only to the old cells, since they do not differ when young.

After obtaining these rough data, we decided to measure the width of the cell in a larger collection of cultures of B.mycoides isolated from various soils of the USSR. Since better contrast of results was obtained on peptone agar, this medium was used in the main work. For the measurements, as usual, we took 18-hour cultures. The living cells were measured by the above method (Table 10).

Since, for obvious reasons, it was impossible to measure the cells of all cultures in a single day, repeated control tests were made to detect any possible deviations in several cultures. This work showed that, if the cultures are grown at the same temperature and the cells are measured at a definite time after inoculation of the medium, the resultant data are in complete agreement. The few discrepancies were of minor importance; in addition, we do not attach much significance to the length of the cell.

As indicated by these data, the cell width in northern cultures (Moscow and Igarka) is almost identical. The deviations are insignificant and can be explained by the presence of cells of different age, even in a preparation made from a young agar culture. In individual cases, the percentage of such /91
thickened cells differs, which leads to certain fluctuations in the resultant mean values. The collections of B.mycoides isolated from southern soils frequently contain cultures with a thickened cell. Thus, especially southern soils contain unlike forms of B.mycoides; we therefore felt it useful to rearrange Table 10 so as to subdivide the analyzed collection in accordance with the type of B.mycoides described by us. A detailed work-up of the materials yielded definite data (Table 11).

Obviously, a certain regularity exists in these figures. Thus, the variants most frequent in the North (ordinary and annular-rugose variants) have a thinner cell. The cell size somewhat increases in the southern transitional /98
variants. Finally, a wide cell is characteristic of the typical smooth variants. In individual cases, there are deviations that do not substantially modify the general conclusion. The first of these forms have a mean diameter of 1.48 μ, the second 1.56 μ, and the last 1.79 μ. It may be deduced from this that the ratio of the diameter of the typical rugose forms to the diameter of the transitional forms is 1.06, while the corresponding ratio in a comparison of the smooth and transitional variants is 1.13. Thus, these quantities approach a coefficient

TABLE 10

WIDTH OF CELLS OF VARIOUS CULTURES OF B.MYCOIDES WHEN GROWN ON PEPTONE AGAR

Culture		Frequency of Encountered Cell Widths, Expressed in Micrometer Divisions														Average Width	Cell Width (in μ)	Range of Cell Length Variation	
		15	16	17	18	19	20	21	22	23	24	25	26	27	28			In Micrometer Divisions	In μ
1		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Igarka	1	—	1	9	19	13	11	6	1	—	—	—	—	—	—	18.8	1.59	30—60	2.4—4.8
	2	—	—	10	11	11	15	2	2	—	—	—	—	—	—	18.3	1.46	30—59	2.4—4.7
	3	—	4	9	13	7	9	6	1	—	—	—	—	—	—	18.6	1.49	40—65	3.2—5.2
	4	—	1	11	18	10	8	2	1	—	—	—	—	—	—	18.1	1.45	35—60	2.8—4.8
	5	—	5	11	16	11	16	4	2	—	—	—	—	—	—	18.6	1.49	40—70	3.2—5.6
	6	—	—	2	15	10	19	3	3	—	—	—	—	—	—	19.6	1.57	35—50	2.8—4.0
	7	—	—	3	10	13	12	8	4	—	—	—	—	—	—	19.5	1.56	35—52	2.8—4.2
	8	2	2	17	17	10	2	—	—	—	—	—	—	—	—	17.1	1.42	40—75	3.2—6.0
	9	—	—	—	14	10	16	1	—	—	—	—	—	—	—	19.1	1.53	45—50	3.6—4.0
	10	—	—	12	29	9	3	2	—	—	—	—	—	—	—	18.2	1.46	52—72	4.2—5.8
Moscow	1	—	—	1	4	6	12	12	14	2	—	—	—	—	—	20.7	1.66	40—70	3.2—4.8
	2	—	2	15	12	13	8	2	—	—	—	—	—	—	—	18.3	1.46	50—60	4.0—4.8
	3	—	—	4	27	14	17	5	—	—	—	—	—	—	—	18.9	1.51	45—60	3.6—4.8
	4	1	4	7	23	9	5	3	—	—	—	—	—	—	—	18.1	1.45	45—65	3.6—5.2
	5	1	8	15	14	7	7	—	—	—	—	—	—	—	—	17.8	1.42	48—68	3.8—5.4
	6	—	4	20	14	7	4	1	—	—	—	—	—	—	—	17.8	1.42	45—70	3.6—5.6
	7	—	3	21	16	7	3	—	—	—	—	—	—	—	—	17.7	1.42	42—71	3.4—5.8
	8	—	5	12	16	12	6	3	2	1	—	—	—	—	—	18.4	1.47	35—60	3.8—4.8
	9	—	8	15	19	11	6	1	—	—	—	—	—	—	—	17.9	1.43	38—60	3.0—4.8
	10	—	8	11	17	10	7	1	—	—	—	—	—	—	—	17.9	1.43	40—70	3.2—5.6
	11	—	—	2	19	10	18	—	—	—	—	—	—	—	—	18.9	1.51	40—65	3.2—5.2
	12	—	—	13	25	5	5	—	—	—	—	—	—	—	—	18.0	1.44	40—50	3.2—5.0

TABLE 10 (cont'd)

Culture		Frequency of Encountered Cell Widths, Expressed in Micrometer Divisions														Average Width	Cell Width (in μ)	Range of Cell Length Variation	
		15	16	17	18	19	20	21	22	23	24	25	26	27	28			In Micrometer Divisions	In μ
1		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Moscow	13	—	—	12	45	14	4	—	—	—	—	—	—	—	—	18.1	1.45	35—70	2.8 — 5.6
	14	—	1	7	30	7	3	2	—	—	—	—	—	—	—	18.2	1.45	42—48	3.4 — 3.8
	15	—	1	12	15	8	9	4	—	—	—	—	—	—	—	18.6	1.49	38—47	3.0 — 3.8
	16	—	1	18	23	6	2	1	—	—	—	—	—	—	—	17.9	1.43	40—60	3.2 — 4.8
	17	—	2	16	17	7	8	3	2	—	—	—	—	—	—	18.4	1.47	39—55	3.1 — 4.4
	18	2	5	8	15	11	4	2	1	—	—	—	—	—	—	18.1	1.45	40—55	3.2 — 4.4
	19	—	—	2	34	8	3	—	—	—	—	—	—	—	—	18.3	1.46	45—60	3.6 — 4.8
Sumy	1	—	—	—	—	19	5	8	27	9	—	—	—	—	—	21.7	1.74	45—60	3.6 — 4.8
	2	—	—	—	—	3	3	20	8	15	—	—	—	—	—	20.7	1.66	50—69	4.0 — 4.8
	3	—	—	—	—	—	9	9	23	4	3	2	—	—	—	21.8	1.75	40—55	3.2 — 4.4
	4	—	—	—	1	3	5	5	23	11	2	—	—	—	—	21.9	1.75	55—56	4.4 — 4.5
Rostov	1	—	—	6	16	13	9	3	1	—	—	—	—	—	—	18.8	1.50	48—55	3.8 — 4.4
	2	—	—	—	2	9	20	24	—	—	—	—	—	—	—	20.2	1.62	42—52	3.4 — 4.2
	3	—	—	—	9	9	20	12	2	—	—	—	—	—	—	20.0	1.60	65—70	5.2 — 5.6
	4	—	—	2	7	15	23	5	—	—	—	—	—	—	—	19.6	1.57	45—50	3.6 — 4.0
	5	—	—	4	5	10	17	7	2	—	—	—	—	—	—	19.5	1.56	45—50	3.6 — 4.0
	6	—	—	2	3	3	11	12	10	4	1	—	—	—	—	20.7	1.66	42—60	3.4 — 4.8
	7	—	—	4	15	16	7	6	1	—	—	—	—	—	—	19.0	1.52	45—50	3.6 — 4.0
	8	—	—	3	14	13	15	1	22	—	—	—	—	—	—	19.1	1.53	40—48	3.2 — 3.8
	9	—	—	—	2	2	14	14	6	—	—	—	—	—	—	20.7	1.66	45—52	3.6 — 4.2
	10	—	—	—	22	20	9	—	—	—	—	—	—	—	—	18.7	1.50	38—48	3.0 — 3.8

TABLE 10 (cont(d)

Culture		Frequency of Encountered Cell Widths, Expressed in Micrometer Divisions														Average Width	Cell Width (in μ)	Range of Cell Length Variation	
		15	16	17	18	19	20	21	22	23	24	25	26	27	28			In Micrometer Divisions	In μ
1		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Frunze	1	—	—	1	20	20	8	1	—	—	—	—	—	—	—	18.8	1.50	45—50	3.6 — 4.0
	2	—	—	1	16	15	12	5	3	—	—	—	—	—	—	19.2	1.54	40—50	3.2 — 4.0
	3	—	—	2	4	5	12	13	11	3	—	—	—	—	—	20.5	1.64	40—55	3.2 — 4.4
	4	—	1	3	3	9	17	9	7	1	—	—	—	—	—	20.0	1.60	45—48	3.6 — 3.8
	5	—	—	2	14	13	17	2	—	—	—	—	—	—	—	19.1	1.53	—	—
	6	—	—	4	16	9	11	3	5	3	—	—	—	—	—	19.4	1.55	38—45	3.0 — 3.6
	7	—	—	1	10	7	22	3	6	—	—	—	—	—	—	19.7	1.58	35—45	2.8 — 3.6
	8	—	—	3	7	5	23	4	1	—	—	—	—	—	—	19.7	1.58	40—50	3.2 — 4.0
	9	—	1	14	13	8	8	4	2	—	—	—	—	—	—	18.8	1.50	43—62	3.4 — 5.0
	10	—	—	6	31	10	3	—	—	—	—	—	—	—	—	18.2	1.45	45—50	3.6 — 4.0
	11	—	—	—	1	4	6	7	18	12	4	2	—	—	—	22.6	1.81	25—45	2.0 — 3.6
	12	—	—	—	2	3	8	9	14	7	7	1	—	—	—	21.7	1.74	48—52	3.8 — 4.2
	13	—	—	—	21	13	10	2	—	—	—	—	—	—	—	18.9	1.51	44—50	3.5 — 4.0
	14	—	—	—	13	3	4	6	15	5	—	—	—	—	—	20.5	1.64	45—50	3.6 — 4.0
	15	—	—	—	6	4	10	8	9	6	—	—	—	—	—	20.7	1.66	45—55	3.6 — 4.4
	16	—	—	—	—	—	5	13	16	7	2	—	—	—	—	21.7	1.74	45—50	3.6 — 4.0
Pakhta-Aral'	1	—	1	3	16	12	11	4	2	1	—	—	—	—	—	19.0	1.52	45—50	3.6 — 4.0
	2	—	—	4	20	10	14	7	4	—	—	—	—	—	—	19.4	1.55	55—60	4.4 — 4.8
	3	—	—	7	17	12	7	4	1	—	—	—	—	—	—	18.7	1.50	45—48	3.6 — 3.8
	4	—	—	2	4	11	9	14	6	3	1	—	—	—	—	19.3	1.54	50—65	4.0 — 5.2
	5	—	—	5	13	8	15	8	1	—	—	—	—	—	—	19.2	1.54	45—55	3.6 — 4.4
	6	—	—	—	11	6	18	10	5	—	—	—	—	—	—	19.9	1.59	35—45	2.8 — 3.6

TABLE 10 (cont'd)

Culture		Frequency of Encountered Cell Widths, Expressed in Micrometer Divisions														Average Width	Cell Width (in μ)	Range of Cell Length Variation	
		15	16	17	18	19	20	21	22	23	24	25	26	27	28			In Micrometer Divisions	In μ
1		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Pakhta-Aral'	7	—	—	—	5	5	17	6	8	8	4	1	—	—	—	21.0	1.68	40—60	3.2 — 4.8
	8	—	—	—	—	—	8	7	15	11	4	—	—	—	—	21.8	1.78	25—30	2.0 — 2.4
	9	—	—	—	—	—	—	2	16	14	14	2	2	—	—	22.5	1.80	45—50	3.6 — 4.0
	10	—	—	—	—	—	—	5	10	15	10	8	5	3	2	23.7	1.90	45—60	3.6 — 4.8
	11	—	—	—	—	—	—	—	—	4	12	21	8	2	1	24.5	1.96	40—55	3.2 — 4.4
Samarkand	1	—	—	8	8	11	10	3	2	—	—	—	—	—	—	19.0	1.52	47—65	3.8 — 5.2
	2	—	—	1	15	19	10	6	—	—	—	—	—	—	—	19.1	1.53	45—50	3.6 — 4.0
	3	—	—	—	8	15	16	5	4	2	—	—	—	—	—	19.8	1.58	45—55	3.6 — 4.4
	4	1	1	6	9	10	14	7	8	1	—	—	—	—	—	19.5	1.56	40—45	3.2 — 3.6
	5	—	—	—	9	17	13	5	—	—	—	—	—	—	—	19.6	1.57	45—58	3.6 — 4.6
	6	—	—	5	13	25	4	—	—	—	—	—	—	—	—	18.9	1.51	45—50	3.6 — 4.4
	7	—	1	9	13	15	7	—	—	—	—	—	—	—	—	18.7	1.50	40—45	3.2 — 4.4
	8	—	—	—	13	13	13	9	—	—	—	—	—	—	—	19.4	1.55	42—50	3.5 — 4.0
	9	—	—	1	13	16	13	5	1	—	—	—	—	—	—	19.2	1.54	40—50	3.2 — 4.0
	10	—	—	6	10	13	18	—	2	2	—	—	—	—	—	19.2	1.54	45—55	3.6 — 4.4
	11	—	1	10	27	11	—	—	—	—	—	—	—	—	—	18.1	1.45	45—50	3.2 — 4.0
	12	—	—	1	11	9	16	5	3	—	—	—	—	—	—	19.5	1.56	35—48	2.8 — 3.8
	13	—	—	—	6	8	7	7	7	7	3	1	2	—	—	21.1	1.69	55—62	4.4 — 5.0
	14	—	—	1	3	11	12	—	5	1	—	—	—	—	—	19.2	1.54	40—50	3.2 — 4.0
	15	—	—	3	8	22	8	3	1	—	—	—	—	—	—	18.9	1.51	45—60	3.6 — 4.8
	16	—	—	—	—	2	2	1	4	10	10	13	6	2	—	23.4	1.87	47—65	3.8 — 5.2

TABLE 11

CELL WIDTHS OF VARIOUS FORMS OF B.MYCOIDES

(Measurement of Young Cells Grown on Peptone Agar)

Variant	Culture		Cell Width, in Micrometer Divisions	Average Cell Width	
				In Micrometer Divisions	In μ
1	2		3	4	5
Usual rugose	Igarka	1	18.8		
		2	18.3		
		3	18.6		
		4	18.1		
		5	18.6		
		7	19.5		
		8	17.7		
		10	18.2		
	Moscow	1	20.3(?)		
		2	18.3		
		3	18.9	18.3 ± 0.5	1.46 ± 0.04
		5	18.1		
		6	17.8		
		7	17.7		
		8	18.4		
		9	17.9		
		10	17.9		
		12	18.0		
		13	18.1		
		14	18.2		
		18	18.1		
		19	18.3		
		16	17.9		
	Frunze	2	19.2		
		9	18.8		
	Pakhta-Aral'	5	19.2		
	Samarkand	6	18.9		
	Igarka	6	19.6		
		9	19.1		
Annular rugose	Moscow	5	17.8	18.8 ± 0.7	1.50 ± 0.06
		11	18.9		
		17	18.4		
	Sumy	1	21.7(?)		
		2	20.2		
		3	21.8(?)		
Strictly rugose	Rostov	1	18.8		
		2	20.2		
		3	20.0		
		4	19.6		
		5	19.5		
		6	20.7	19.6 ± 0.8	1.57 ± 0.07

Variant	Culture		Cell Width, in Micrometer Divisions	Average Cell Width	
				In Micrometer Divisions	In μ
1	2		3	4	5
Strictly rugose	Rostov	7	19.0		
		8	19.1		
		9	20.7		
		10	18.7		
	Frunze	10	18.2		
	Pakhta-Aral'	1	19.0		
		2	19.4		
		7	21.0		
	Samarkand	3	19.8		
		12	19.5		
		13	21.1		
		16	23.4(?)		
Transitional (elliptical variant)	Frunze	1	18.8		
		4	20.0		
		6	19.4		
		14	20.5		
		15	20.7	19.5 ± 0.65	1.56 ± 0.05
	Pakhta-Aral'	3	18.7		
		4	19.3		
	Samarkand	5	19.6		
		9	19.2		
		14	19.2		
		15	18.9		
Transitional (elliptical plumose variant)	Moscow	15	18.6		
	Sumy	9	21.9		
	Frunze	3	20.5		
		5	19.1		
		7	19.7		
		8	19.7		
		11	26.6(?)	19.3 ± 0.68	1.54 ± 0.05
		12	21.7(?)		
		13	18.9		
	Samarkand	1	19.0		
		2	19.1		
		5	19.5		
		7	18.7		
		8	19.4		
		10	19.2		
		11	18.1		
Smooth	Pakhta-Aral'	6	19.9		
		8	21.8		
		9	22.5		1.79 ± 0.07
		10	23.7		
		11	24.5		
	Frunze	16	21.7	22.4 ± 0.93	

of 1.10.

On statistical work-up of the material, these differences may be found as unreliable. This formal consideration, however, is convincingly refuted by the character of the shifts obtained in measuring the cells of B.mycoides forms of different type.

An analysis of the figures in Table 11 clearly shows that the working surface of relatively young cells of various forms of B.mycoides, grown on peptone agar, will be far from identical. Thus, if a cylindrical shape is arbitrarily adopted for the rod of B.mycoides, then the surface of the cells of the rugose variants will be 22.1 μ, while that of the smooth variants will be 27.7 μ. The intermediate forms will have a surface of 23.5 μ (for a rod length of 4.0 μ).

In 1946, Rautenshteyn published a paper in which he also came to the conclusion that cultures of B.mycoides isolated from southern soils have a thicker cell. A certain divergence between the absolute figures given by Rautenshteyn and those obtained by us can possibly be explained by the fact that different nutrient media were used in the experiments.

It is well known that, in the same species of animals, on passage from North to South, the body size diminishes. This is explained by the defense reaction in the struggle for heat economy. We have the opposite relationship in the ecotypes of B.mycoides. It might well be that, in aerobic bacteria grown at elevated temperatures, the increase in active cell surface stimulates a better assimilation of oxygen which is poorly soluble in such a medium. Future studies will show the degree of generality of this phenomenon.

On MPA, as was to be expected from the above discussion, the cell size of all cultures of B.mycoides studied at a young age was almost identical. A similar picture was obtained on a medium containing 1% peptone. Thus, on all rich media young cells of all B.mycoides forms are of the same size (Table 12).

Since we are here speaking of an increase in cell width with age, it is /100 not without interest to indicate the width of the spores of the different variants of B.mycoides (Table 13). It will be seen that the spore width of the intermediate variants is identical with that of the ordinary rugose variants. In the smooth form of B.mycoides, the spores are somewhat larger.

Comparing the width of a cell of B.mycoides with that of the spores, it is easy to conclude that the thickening of the aged cell cannot be explained by its mechanical stretching by the spore formed.

In conclusion, a few statements should be made on the length and form of B.mycoides cells. In most variants, the cells are cylindrical and only in the smooth variants do they acquire an ovoid form. The cell length does not differ substantially among the different variants but, according to our impression, the smooth variants tend less to elongated cells.

This permits the conclusion that the cell sizes differ slightly among the various forms of B.mycoides.

TABLE 12

CELL WIDTHS OF VARIOUS CULTURES OF B.MYCOIDES
(Measurement of Young Cells Grown on MPA)

Culture		Frequency of Encountered Cell Widths, Expressed in Micrometer Divisions									Average Cell Width	
		15	16	17	18	19	20	21	22	23	In Micrometer Divisions	In μ
Igarka	4	—	4	5	17	11	—	—	—	—	18.1	1.45
	6	1	10	24	20	—	1	—	—	—	17.2	1.33
	7	1	9	22	15	2	—	—	—	—	17.2	1.38
	10	1	3	24	18	3	—	—	—	—	17.4	1.39
Moscow	1	2	15	17	13	2	—	—	—	—	17.0	1.36
	10	2	22	14	11	2	2	—	—	—	16.9	1.35
	12	3	16	18	13	3	4	—	—	—	17.1	1.37
	15	—	—	15	25	8	6	5	1	—	18.4	1.47
	16	4	11	24	26	13	4	1	—	—	17.5	1.41
Sumy	1	5	14	15	13	1	—	—	—	—	16.8	1.34
	2	3	9	19	15	3	—	—	—	—	17.1	1.37
	3	—	7	19	19	2	—	—	—	—	17.3	1.38
	4	—	—	23	11	6	2	—	—	—	17.7	1.42
Rostov	5	2	16	29	6	1	—	—	—	—	16.8	1.34
	6	3	12	20	13	—	—	—	—	—	16.9	1.35
Frunze	2	—	—	14	23	10	—	—	—	—	17.9	1.43
	3	—	4	24	15	3	—	—	—	—	17.3	1.38
	4	2	20	16	9	5	1	1	—	—	17.0	1.36
	9	4	12	23	10	1	—	—	—	—	16.8	1.34
	10	6	15	25	3	3	1	—	—	—	16.7	1.33
	13	—	6	23	12	2	—	—	—	—	17.2	1.38
	15	3	14	15	10	3	2	—	—	—	17.0	1.36
	16	—	—	—	18	16	6	3	—	—	18.9	1.51
Pakhta-Aral'	4	—	4	12	26	4	—	1	—	—	17.8	1.42
	5	—	1	13	23	6	2	—	—	—	16.9	1.35
	9	—	6	8	16	8	8	3	2	—	18.4	1.47
	10	—	—	1	28	13	15	9	2	2	18.3	1.46
	11	—	5	24	15	2	2	—	—	—	17.4	1.39
Samarkand	2	5	25	26	3	—	—	—	—	—	16.5	1.32
	3	4	21	10	8	—	—	—	—	—	16.5	1.32
	4	3	12	18	10	7	1	—	—	—	17.2	1.38
	14	1	9	22	17	5	—	—	—	—	17.3	1.38
	15	—	1	20	20	5	1	—	—	—	17.7	1.42
	16	1	10	21	17	3	2	—	—	—	17.3	1.38

TABLE 13

SIZE OF SPORES IN DIFFERENT VARIANTS OF B.MYCOIDES

Culture	Variant	Spore Width	
		In Micrometer Divisions	In μ
Igarka, No.5	Ordinary rugose	13.7	1.10
Same, No.6	"	13.4	1.07
Moscow, No.15	Intermediate	13.0	1.04
Golodno Step', No.8	"	13.8	1.10
Same, No.10	Smooth	15.0	1.20
Same, No.11	"	15.1	1.21

Earlier, we stated that laboratory cultivation causes a change in the diameter of the B.mycoides cell and that we isolated about 20 typical rugose forms from Moscow soils. The cultures were isolated on the same type of medium as used by Novogrudskiy and Kononenko. For full reproduction of their working conditions, the preparations were measured after staining with Manson's stain. The entire analyzed collection was found to have closely adjacent cell diameters, characteristic of the rugose forms of B.mycoides. However, as noted above, /101 the young cells, even of smooth variants of B.mycoides on a 1% peptone medium, have cell diameters identical with those of the rugose forms.

Thus, also this experiment yields a negative answer to the question whether cultures of different cell widths exist among certain variants of B.mycoides.

Our own experience permits the following statement:

1. Novogrudskiy and Kononenko were correct in noting the existence of B.mycoides forms with different cell widths. In our opinion, however, cultures with a different cell width do not necessarily exist within the bounds of a single variant. According to this characteristic, only cultures taken from different variants will differ.

In establishing the cell size in B.mycoides, the ontogenesis must be taken into consideration.

The composition of the nutrient medium also has some influence on the cell width.

2. During the process of aging of the cells of all B.mycoides variants, appreciable thickening takes place.

On MPA, the cell size of all variants is equal when they are young. On aging, however, the cells of the rugose variants thicken ~16% of their original width, while the cells of the smooth variants thicken by 35%. The transitional forms occupy an intermediate position.

On poor media (agar with 0.1% peptone), the cell width of different variants of B.mycoides differs even in youth.

Cell thickening is preceded by formation of a prosporangium.

3. Bearing in mind the changes of the cell during ontogenesis, it may nevertheless be asserted that the various subspecies of B.mycoides differ among themselves in cell width. The thinnest variants, which are predominant in the North (ordinary and annulo-rugose), are followed in this respect by the southern forms (strictly rugose and elliptical). The widest cell is encountered in the smooth forms of B.mycoides. The numerical ratio of the diameters of the principal B.mycoides forms is close to 1.10 - 1.15.

4. The increase in cell surface in the southern forms of B.mycoides can be explained by adaptation, which promotes better assimilation of oxygen whose solubility in water at elevated temperature is relatively poor.

5. The differences in cell size between the individual forms of B.mycoides are not sufficiently great to construct a system of identification exclusively on this basis.

These differences become significant only when other culture features are taken into account, specifically the colony structure.

2. Cell Structure of B.Mycoides /102

B.mycoides often has been selected as the object for various studies on the structure of bacterial cells, because of the relatively large cell size. Unfortunately, to date there are no comparative tests available on the structural features of the cells of B.mycoides variants.

Nevertheless, we considered it useful to generalize the existing data on this subject, since certain features of the B.mycoides cell are highly characteristic and may be used for its identification.

It should be mentioned that numerous authors, in studying the question of the existence of a nucleus in bacteria, experimented with B.mycoides.

This question has been answered differently by different authors. Thus, Varlikh (1891), on the basis of his study of a number of bacteria including B.mycoides (B.pseudoanthracis), concluded that the cells of most bacteria consist of nuclei surrounded by envelopes and do not contain cytoplasm.

Some authors, such as Penau (1912), postulated the existence of a diffuse nucleus in spore-bearing bacteria, including B.mycoides, with a certain state of the chromidial nucleus corresponding to each stage of the development of rod forms.

Kruiss (1913), who made photomicrographs of B.mycoides under ultraviolet light, found formations of rounded shape in the cell, which he considered to be nuclei.

Pitschmann and Rippel (1932) excluded the existence of a cell in bacteria on the basis of certain theoretical calculations. At the same time, using the Feulgen reaction, they found that B.mycoides does have a nuclear substance but that this is in a diffuse state and has no structure, which facts would lead to its uniform distribution between the cells on their division.

The existence of an autonomous nucleus in B.mycoides, as is the case in a number of other bacteria, was advocated by Paravicini (1918) and by Holland (1931 - 1934). In Holland's opinion, the bacteria have a complex nucleosomal apparatus whose interrelation with the other cell constituents varies constantly during ontogenesis.

Badian (1933 - 1935) postulated the existence of a constant number of chromosomes in spore-bearing bacteria, including B.mycoides. In his opinion, they undergo fission, reductive division, autogamy, etc.

Imshenetskiy (1940), in attempting to answer the question as to the existence of a specific nuclear substance (thymonucleic acid) in the bacterial cell, determined it by Feulgen's reaction in bacterial precipitates. Among other bacteria, he also investigated B.mycoides. He was able to demonstrate the presence of this substance, which is most specific for chromatin, in the bacterial masses, and also found that the content of thymonucleic acid was greater in the bacterial cell than in lower and higher plants. /103

The high content of nucleoproteins and thymonucleic acid in the bacterial cell must not be associated with the presence of an avisual nucleus. The calculations by Imshenetskiy for B.mycoides show that the nucleus in its cell should be of the same size as the spore, i.e., about 0.8 μ. However, even from careful cytological observations he did not feel justified to assume chromatin structures analogous to a nucleus as found in the bacterial cell. He believed that many authors took lipoprotein bodies, polar granules, and other cellular formations for a nucleus. In normal environment, a segregation of chromatin structures is observed during the germination of spores, after which the nuclei again are dispersed into the chromatin granules.

In many cases, morphological differentiation of nuclear substances in bacteria also occurs, under the influence of anomalous living conditions and associated degeneration. Most often the formation of such "nuclei" is observed in giant cells.

Several investigators have described a "spiral" nucleus or a "nucleus of spirogyra type" in spore-bearing bacteria. These spiral structures are entirely absent from young vegetative forms, that have just sprouted from spores. Such structures form at a time at which the most intense bacterial multiplication has ended. Their presence indicates a relatively advanced age of the cell.

The appearance of a spiral structure in cells of spore-bearing bacteria has been described in Imshenetskiy's monograph "The Structure of Bacteria". Here, we merely note that filamentous structures may be detected in living unstained cells, which means that they cannot be considered as being artifacts (Fig.65).

Judging from the microchemical reaction, the constituent substance of the filament does not contain thymonucleic acid.

The exceptional strength of the filament is a striking phenomenon. It does not break even after boiling in water for 10 min, and does not dissolve in ether, alcohol, acetic acid, 1% NaOH, 1% H_2SO_4, or other similar substances.

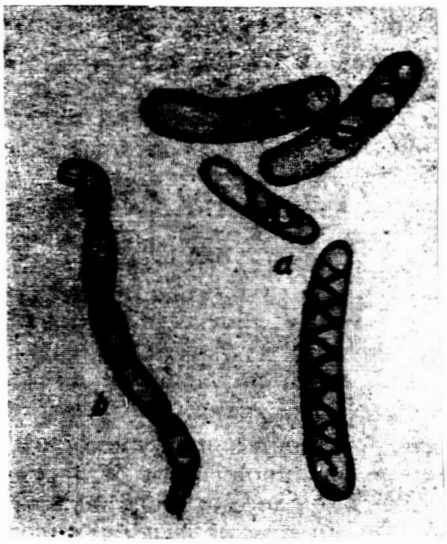

Fig.65
a - Formation of filamentary structure in the cell of B.mycoides; b - Lipoprotein inclusions in the B.mycoides cell (according to Imshenetskiy)

Spiral formations are also encountered in a number of bacteria with a /104
differentiated nuclear apparatus. These considerations induced Imshenetskiy to consider the filamentous structures as cytoplasmatic formations and to reject the assumption that they are nuclear substances. The reserve substances of the cell do not participate in their formation.

Speaking of the nucleus in bacteria (including B.mycoides), it should be noted that several authors were able to demonstrate the existence, in these microorganisms, of structurally formed nucleotides, homologs of the nuclei of higher organisms (Peshkov, Robinow, Klieneberger, and others). Apparently, this question will soon be elaborated further.

Lipoprotein bodies can be found in the B.mycoides cell. Their presence has been noted by various authors. Thus, Stapp and Zycha (1931) encountered bodies of a lipoid nature in cells of B.mycoides grown on several media. These authors postulated that such bodies resemble the "sporoid" formations described by Růžicka (1908). They were completely insoluble in ether, chloroform, and benzine. Nevertheless, some of our own observations make us believe that /105
these bodies are of the lipoid type.

The existence of fat in the B.mycoides cell has been established by Sparrow (1933), using the Meyer-Eisenberg microchemical method. Parallel to this, he also made a series of quantitative determinations.

Similar observations were obtained by Lewis (1932). The addition of various sugars to the medium increased the yield of bacterial mass but decreased the fat production. During less vigorous multiplication of the cells, the fat accumulation apparently is more extensive.

Sparrow postulates that fat formation is more likely due to catabolism than to synthesis of reserve substances.

Imshenetskiy studied their behavior in detail in the cell of spore-bearing bacteria during ontogenesis (Fig.65).

He was able to demonstrate that these granules have the characteristic of reserve substances, since any change in cultural conditions resulted in their decrease or increase.

On strong increase in the volume of lipoprotein bodies, the interconnecting filaments undergo extreme shortening and change into a kind of dumb-bell shape. Generally, one pair of bodies is formed in the cell, less often two or three pairs. At first, these occupy an oblique position but then arrange themselves along the longitudinal axis of the cell.

Cells were encountered whose contents consisted almost entirely of large glistening inclusions of spherical shape. When such cells were transferred to a fresh medium, the size of the lipoprotein bodies decreased and the cells resumed active division. It can be concluded from this that any excessive accumulation of lipoprotein bodies must be considered as a degenerative but reversible phenomenon.

The lipoprotein bodies, according to data by Imshenetskiy, are soluble in acetone, acetic acid, and particularly in lactic acid, but insoluble in ether, chloroform, toluene, and alcohol. They exhibit considerable resistance to heating, and withstand five minutes boiling in water, maintaining their spherical form and characteristic luster during such boiling.

The outward appearance of the lipoprotein bodies may be a reason for confusing them with the chromatin formations resulting in amitotic cell division; smaller granules of the same type may be taken for chromatin bodies if they are more readily stained by the usual stains. Such errors have been repeatedly made by various authors (Averinzew, Holland, and others).

It can finally be pointed out that Stapp and Zycha noted the presence of volutin in normal cells of B.mycoides.

3. Degenerative Cell Changes /106

Under the influence of various factors, the B.mycoides cell often assumes an atypical form. Usually, such phenomena are associated with degeneration of

the culture.

In this Section, we will give a general idea on the B.mycoides cell of unusual form and, as far as possible, define the causes for their appearance.

We will list the papers devoted to atypical cells of B.mycoides in chronological order.

Apparently, the first to describe the involutional cells of B.mycoides was Varlikh (1891), who noted their appearance in aged cultures. Elevated temperatures also caused the appearance of atypical cells. These cells were distinguished by smaller size, a rounded form, and the absence of chromatin. Their contents had a homogeneous form.

Cells so altered died rather soon.

This question, with respect to B.mycoides, has also been studied by Nadson et al. (1910, 1934), Oesterle and Stahl (1930), Stapp and Zycha (1931), Lewis (1932), and other authors and, lastly, has been elegantly solved in Imshenetskiy's monograph (1940).

We will present the principal conclusions of these authors:

The most detailed early studies of abnormal cells in B.mycoides were those by Nadson and Adamovich (1910) who found that, in media to which thermostable metabolic products of B.mycoides had been added, the external habitus of a culture of this microorganism was substantially modified. The cells elongated and began to resemble the hyphae of actinomycetes. The capacity to sporulate was lost, and the cells usually were surrounded by a capsule which evidently had a protective purpose.

In 1916, Smirnov noted a lag in the growth and sporulation of B.mycoides under the influence of high NaCl concentrations. He observed a decrease in spore size and a reduction in thermostability of the spores.

Subsequent studies took place at a much later date.

Oesterle and Stahl (1930), who agreed with the Löhnis' view as to the life cycle of bacteria, used results of their work as basis for outlining the regular changes of form of B.mycoides cells. They studied three cultures isolated from soils. Since, when using standard media, these authors ordinarily obtained /107 cells of stable form, they cultured B.mycoides in soil extract, in extracts of putrefacient substances, and in plain broth irradiated by sunlight or ultraviolet rays.

Various concentrations of NaCl, mercuric chloride, soda, and chloramine were also added to the nutrient media.

Under the action of all these materials, a wide range of cell modifications occurred. The media were found to contain rounded cells, granulating disintegrated cells, filamentous formations, swollen "gonidia", pyriform cells, small gonidia, an amorphous mass of "symplasm", etc.

In a number of nutrient media, the authors noted filtrable forms of B.mycoides which, when transferred to fresh nutrient media, gave atypical colonies consisting of gram-negative cocci.

In a certain environment, the atypical forms reverted to the original culture of B.mycoides. The reversion of atypical cells to normal cells depended on many conditions. For example, the passage of "gonidia" into the typical forms seemed to proceed more rapidly in the spring and summer months than in the winter.

This transformation was sometimes sudden, but occasionally passed through a series of intermediate forms.

In the opinion of Oesterle and Stahl, all these transformations fit into the scheme of life cycles described by Löhnis, which - as is well known - was based entirely on inaccurate and erroneous observations.

In many cases, Oesterle and Stahl were dealing with actually modified cells of B.mycoides. However, there is no doubt that they worked with contaminated cultures, so that is is difficult to distinguish reliable observations from the erroneous ones. The assertion that filtrable forms, symplasms, gonidia and gonidangia exist in B.mycoides, must be considered incorrect since such formations were never encountered by later authors.

Nevertheless, some authors (Schmidt and others), who studied the development of B.mycoides, support the views of Oesterle and Stahl.

Stapp and Zycha (1931) did detailed work on B.mycoides. They assume that the transformation of the spore into the rod and of the rod back into the spore is the normal cycle of development of B.mycoides.

In a broth culture of B.mycoides, the gradual change of some of the cells can be noted. They become fewer, lose their refractility and their stainability. Cell residues and highly granulated forms can be observed in the medium. Parallel to this, the bacterial mass becomes slimy (formation of "symplasm"?). Some cells attain a large size, with formation of spherical bodies at their /108 interior that occupy the diameter of the entire cell and often take on a cylindrical form.

However, a similar picture can also be observed on other media, especially on addition of 2 - 5% glycerin.

These formations within the cell were observed by Růžicka (1908), who took them for "sporoidal bodies".

Stapp and Zycha believed that they were of a lipoid nature. They were unable to obtain complete solution of the bodies in ether, chloroform, or benzine. Nevertheless, a number of arguments speak for the lipoid nature of this formation.

Nyberg called them "endoplasts", but did not further define their nature.

On loss of their normal state, the cells no longer stain properly with Gram's stain. In addition, faint staining or staining of individual regions is observed.

In the dark field, the normal cells are luminescent, with a bright shiny membrane. The cell contains luminescent granules. On metamorphosis of the cell, the membrane becomes irregularly thickened, and the cell contents coagulate. "Fatty" drops give strong luminescence and can be distinguished from the spores by their irregular outline.

A two-day culture grown on MPB will have only normal cells. Atypical forms appear only later. With time, normal cells in the medium are found only as exceptions.

To determine the viability of atypical cells, Stapp and Zycha isolated 50 cells by means of a micromanipulator. Not one of them was capable of further multiplication whereas, of 45 isolated cells with normal appearance, 75% exhibited growth.

The transfer of a culture containing only atypical cells to a fresh medium was likewise unsuccessful; no growth was obtained.

These experiments convincingly demonstrate the degenerate nature of the cells with modified shapes.

Stapp and Zycha subsequently investigated the influence of various salts on the B.mycoides cell. The experiments were made on MPB media diluted with water to double the quantity. They defined the critical molar concentrations of various salts at which growth of B.mycoides could still take place.

It is of interest that only the addition of magnesium salts caused the appearance of long filaments and round vesicular forms. All magnesium salts caused the formation of such cells, 3 - 9 μ in diameter, recalling the "gonidangia" of other investigators. Nyberg termed these cells "cysts". In the /109 author's experiments they never showed capability for further multiplication.

All cultures of B.mycoides yielded vesiculose cells in the presence of magnesium salts, while other spore-bearing and non-spore-bearing bacilli did not show this tendency.

Stapp and Zycha recommend the use of this specific property as a determinative trait (Fig.66).

A medium of MPB with 12.4% $MgSO_4$ (0.5 mol. solution) can be used to observe this phenomenon. After several days, large swollen bodies, reaching a size of 9 μ, are formed together with the normal cells in a B.mycoides culture. Sometimes, these bodies are hyaline and sometimes they contain round inclusions. In the dark field, the membranes of the large cells stand out well, and the round inclusions strongly refract light. Except for the latter formations, the entire cell has good affinity for aniline dyes.

This reaction to magnesium salts was not considered characteristic by

Imshenetskiy, since cells of other spore-bearing bacteria may also yield similar formations. B.mycoides, however, modifies its cells at considerably lower concentrations of magnesium salts, and therefore we agree with Stapp and Zycha on this point.

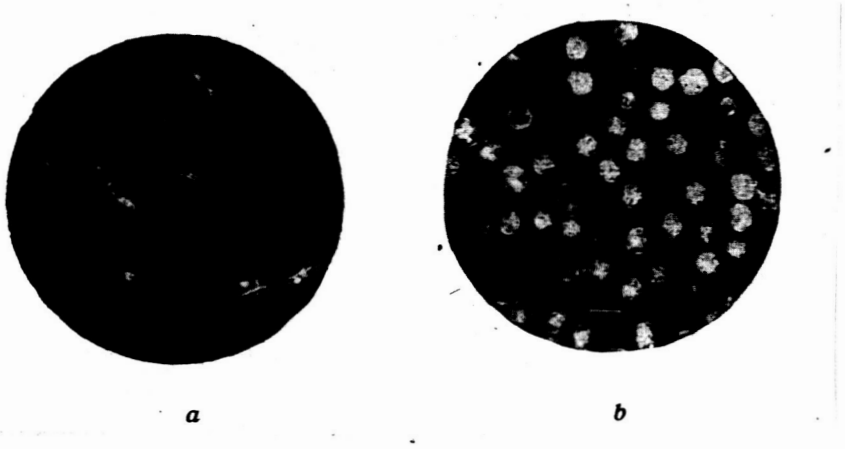

Fig.66 Formation of Vesiculose Cells in a Culture of B.Mycoides. Dark-Field Photomicrograph (according to Stapp and Zycha) a - Normal rods of B.mycoides; b - Vesiculose cells of B.mycoides

In old cultures, a considerable portion of the round cells disintegrate. The membrane breaks, and the contents of various cells often become confluent.

In media containing $MgSO_4$, the spores of B.mycoides germinate normally, /110 but cell division is reduced and filaments as long as 400 μ are formed. The cells often swell, and the formed spherical bodies of a granular structure may separate from the filament. A total of 1 - 3 hours is required for the formation of vesiculose cells. In some cases, spheroidization results from the rounding of individual cells. The size of the round cell is at least 1.5 times the thickness of the daughter cell, and in some cases its diameter may reach 20 μ.

The individual cultures of B.mycoides give vesiculose cells somewhat differing in appearance. Some are hyaline, while in other cases they appear to be filled with round inclusions. These formations, like the grains in degenerate cells, apparently consist of lipoid substances. They cannot, however, be completely dissolved in ether.

On addition of glycerol to the medium, the formation of lipoid bodies increases. A brown lipoid mass is extracted from the centrifugate of a culture, after drying and triturating with quartz sand, by petroleum ether, alcohol, or benzine. Chloroform also dissolves a certain waxy substrate. The ether extract is completely saponified by KOH. This confirms the view that these spheres are rich in fat.

To verify the viability of the swollen cells, 102 of them were isolated by

means of the micromanipulator. Under hanging-drop conditions, they yielded no growth. This would mean that this formation consists of degenerate bodies.

The work of Lewis (1932), likewise on the morphological variability of B.mycoides, was done very carefully. He was primarily concerned with the fact that, according to Henrici (1928), three types of cells could be found in the bacterial culture: embryonal, mature, and gerontic. Dominance of one form or the other depends on the age of the culture. The term "cytomorphosis" was proposed to denote the changes with age.

Thanks to the ability of B.mycoides to form chains, the age change of its cells is easy to follow. The young cells appear longer and thinner than the old ones. They contain either none or only few granules that stain readily by fuchsine, thionine, or gentian-violet. No differences in the Gram-stainability were noted.

On media unfavorable to sporulation, dead cells were found among the mature ones.

Mature cells usually contain 2 - 5 vital-staining and strongly refractile granules, of a lipoprotein nature. /111

These granules, which are generally present in mature cells, become particularly noticeable in the presence of sugar or glycerin in the medium. In these cases, sporulation is inhibited and the granules may be mistaken for spores. Sometimes the protoplasm divides into a number of nodules located at the poles or in the interstices of bright vacuolate bodies.

The embryonal and mature cells are more intensely stained at the poles. For B.mycoides, this phenomenon was described earlier by Amato (1909).

Under favorable conditions, the cell ultimately sprouts a spore. In this case, if sporulation is retarded by any factors, senile cells (dying out) are obtained.

Many adult cells die without becoming gerontic cells. In old cultures, however, pleomorphic cells are more often encountered. This is particularly true in asporogenic cultures, for example in a medium containing sugar or a medium with a high liquid column, where sporulation is inhibited.

According to Lewis' data, B.mycoides on degeneration may yield atypical cells: long yeast-like, thick bacillary, spiral, mycelioid, etc.

The formation of coccoid cells is easily observed in B.mycoides. They are formed as terminal buds, or appear on repeated division of typical bacillary cells. The buds may be produced by either normal or swollen cells. From their genesis, the cocci are correctly termed "pseudococci". The cocci can divide to give either streptococci or isolated cocci. These forms are not entirely spherical. The author never observed the formation of cocci from the side branches.

The cocci are gram-positive and are readily stained by the usual stains.

The absence of granules and their stainability characterize them as embryonal cells. All attempts to obtain a B.mycoides culture consisting of cocci have been unsuccessful, since the ordinary cultures were produced from the cocci on a fresh medium.

The yeast-like cells often predominate in old cultures. They have been encountered specifically in the mucoid strains growing on sugar-containing media. They may be linear, spherical, or elongated. These cells are often met in pairs, as though coupled after division. Sometimes they form budlike shoots. Such cells were apparently of the type termed "gonidangia" by Löhnis (the /112 zygotes, asci, or "chlamydospores" of other authors). These cells stain vitally and are gram-positive. The author was unable to demonstrate the abscission of small bodies from these cells, despite his great experience. In their structure (staining, granules) they resemble embryonal cells rather than adults. Yeast-like cells, transferred to a fresh medium, grow into typical cells. In the author's opinion these cells can be more aptly termed chlamydospores. However, the membrane of such a cell is not thickened and the cell lacks the structure characteristic of the chlamydospores of fungi.

Large, linear, and spiral cells are infrequently encountered but are always found in old cultures. Long cells appear when cell division is retarded for some reason. Circinate forms are more often found in the interior of the agar than on the surface.

Mycelioid forms of B.mycoides are encountered. These are greatly similar to the hyphae of fungi. It has not been possible to isolate a culture yielding only a mycelioid growth.

Observations by Lewis, on a large amount of material, still did not prove conclusively the existence of a symplasmatic stage or gonidia in B.mycoides.

According to Lewis' findings, B.mycoides also does not form filtrable forms. This was also confirmed by den Dooren de Jong and other authors.

Thus, the most careful investigations have shown that, in old cultures, the cells of B.mycoides may be greatly modified, assuming coccoid, swollen, filamentous, yeast-like, and other forms. This type of transformation must be considered as degenerate, due to the prolonged existence of the cells in a medium with high metabolite content.

Such cells often lose their ability to multiply (Stapp and Zycha) but often remain viable, as shown by Lewis.

The appearance of degenerate cells, when exposed to non-contacting metal plates, was observed by Shtern and Kriviskiy (1932). The influence of the metal became manifest only if they were brought close to the surface of the medium on which the B.mycoides culture was grown (not farther away than 0.5 - 1.0 mm).

In the presence of metal, the cells of B.mycoides began to swell, increased in refractivity, acquired a finer granulation, and exhibited numerous small fat globules in clusters.

Heavy metals suppressed sporulation, evidently due to the involution of /113 the cells of the culture.

In order of their atomic numbers, and in sequence of their action, the metals can be arranged as follows:

$$Pb\,(82) > Au(79) > Pt\,(78) > Mg\,(12).$$

Extensive structural changes of B.mycoides cells under the action of ultraviolet rays were observed by Nadson and Shtern (1934). After irradiation of 2 - 3 min, vacuoles appeared in the cell plasma. The plasma became less transparent and finely granular; a first appearance of a few extremely minute fat globules (lipoid bodies) was later followed by an increasing number of such droplets.

Stronger irradiation inhibited cell division, and the cells assumed clearly involutional forms (dwarf and coccoid cells).

In some cells the plasma slid away and separated from the cell wall; later autolysis of the cell contents took place. Sometimes, it was the cell wall itself that dissolved, leaving a naked protoplasm, similar in form to the "symplasm" of Löhnis.

Brief irradiation of a culture accelerated sporulation, while longer irradiation completely suppressed it.

Borodulina (1935), in studying the interrelation of soil actinomycetes with B.mycoides, noted inhibition and modification of growth of B.mycoides under the action of the toxic products of the actinomycetes. In a fatty medium, containing metabolic products of the actinomycetes, the B.mycoides forms long filaments. The division into cells is greatly retarded. After 17 days in the medium, the formation of normal rods is accompanied by strongly deformed cells which usually are thickened in the middle, or swollen at one end and slightly curved at the other. These cells are 2 - 3 times as long as the normal rods. Here again, large rounded yeast-like cells are encountered. Some of these cells may have one or two vacuoles.

The toxic effect of the metabolic products of actinomycetes showed in an inhibition of sporulation. In individual cases, when B.mycoides was cultured on media containing such toxic products, asporogenic variants of B.mycoides were obtained, forming smooth transparent colonies on mineral-peptone agar, with branchings inside the agar.

Large amounts of the toxic products of actinomycetes stopped sporulation completely, while lower amounts merely retarded it.

The modified (degenerate) cells of B.mycoides lack the capacity for /114 sporulation. However, they must be recognized as viable, since they are able to proliferate on agar media to form typical colonies.

The change in the form of the B.mycoides cell under the action of actinomycetes which are antagonists of these bacteria, was described by Nakhimovskaya (1937) who noted that the cells of B.mycoides colonies, grown in the immediate vicinity of an actinomyces or on a nutrient medium to which the filtrate of an aqueous infusion of agar cultures of actinomycetes had been added, were always more granular and disintegrated sooner than cells of normal colonies.

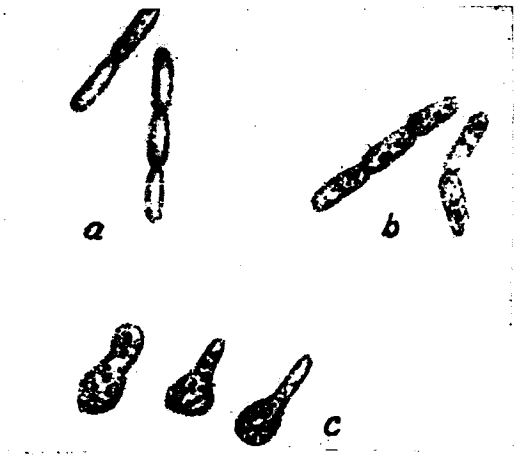

Fig.67 Modification of Cells of B.Mycoides under the Influence of the Antagonistic Action of Actinomycetaceae (according to Nakhimovskaya)
a - Cells grown on MPA, at a distance from a streak of actinomycetes; b - Cells grown on the margin of the sterile zone; c - Cells from cultured material persisting in the sterile zone

The cells of a normal culture of B.mycoides when introduced into the filtrate of a medium containing an actinomyces antagonist, show considerable granulation in only three hours. The disintegration of the cell, with formation of a mass of debris, begins later.

Under the influence of metabolic products of an actinomyces, the B.mycoides cells often assume a swollen rounded or yeast-like form (Fig.67).

Nakhimovskaya notes that a similar degenerative modification of B.mycoides cells can also be found in normal bacterial cultures. Here, however, such /115 modifications proceed much more slowly.

An actinomyces or an extract of its culture never destroys all the microbes of the culture. Even a protracted stay of cells in a filtrate of antagonists does not cancel the viability of all cells of the culture.

Figure 68 clearly shows the antagonistic effect of a culture of Actinomycetaceae on B.mycoides, when these microorganisms are cultured together on Petri dishes.

Novogrudskiy, Kononenko, and Rybalkina (1936) obtained interesting data on the possible degenerative modifications in B.mycoides. They studied the question of the variation of soil-inhabiting bacteria. In their experiments, live bacteria were applied to the surface of small glasses which were buried in the soils to be studied, for various periods of time.

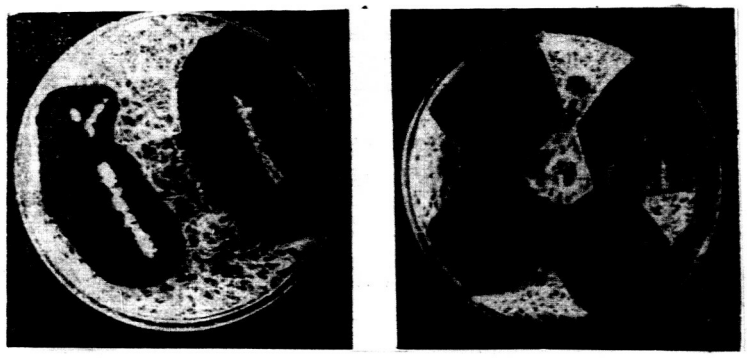

Fig.68 Antagonistic Influence of Actinomycetaceae on the Development of a B.Mycoides Colony (according to Borodulina)

In sterilized soil, disintegration of a considerable number of cells begins within 24 hours. Their form often becomes thickened and globose. The cell contents become granular and are only faintly stained by erythrosine. Some parts of the cell interior are not stained at all. Other cells, however, maintain their normal habitus.

During the next few days, the number of typical cells decreases furthermore. Chains and threads of filaments disappear completely. Moreover, to some extent, the number of disintegrated cells increases.

From about the fifth day, mass destruction of the cells takes place. On the glasses, all succeeding stages of this process can be seen, up to transformation of the cell into clusters of granular material, sometimes following the /116 marginal configuration of the cell on the glass. The impression is gained that, in some cells, the membrane is destroyed first and in others, the cell contents. Cells of the latter type look empty.

The situation in nonsterile soil is entirely different. Cells of B.mycoides in these experiments multiply normally and form long chains.

Sterilization of the soil, as generally known, causes the formation of organic substances having a toxic effect on microorganisms. Evidently, under their influence, normal multiplication of a B.mycoides culture becomes impossible. The alterations undergone by a B.mycoides cell in sterile soil must be considered degenerate for obvious reasons. In essence, these changes are very similar to those described by other investigators.

Imshenetskiy placed special emphasis on the study of atypical cell forms

of spore-bearing bacteria. He found that B.mycoides - like other sporulating bacteria - when grown on media containing 0.5 - 1.0 mol solutions $MgSO_4$, NaCl, and LiCl, will form sharply altered cells.

Small globular cells are the first to appear in the culture. Their number varies from 5 to 30% of the total number of cells in the preparation. The coccoid cells are formed from the rods by shortening and subrounding. When transferred to a fresh medium, the coccoid cells revert to the bacillary form.

The globular cells are more permeable to dyes.

The coccoid cells, gradually increasing in size, may reach substantial dimensions. Thus, in B.mycoides the diameter of a "vesiculose" cell is 8 - 10 μ, and its total volume may be as much as 60 times the average volume of the normal vegetative cell.

The giant cells have pale lusterless contents, with single or multiple vacuoles forming gradually. The vacuolization of the cells is a sign of necrobiotic changes and is evidently connected with the excessive penetration of water into the cell.

In the giant cells, small glistening granules appear immediately beneath the membrane. At times, these granules push out the membrane, causing the topography of the cell to become irregular. Such small granules have often been considered to be gonidia, formed in gonidangia. However, a series of recent experiments showed no germinating capability of these so-called "gonidia".

The extensive stretching causes the membrane of giant cells to thin out /117 greatly. With increasing intracellular pressure, the cells may form shoots resembling pseudopodia.

Such "giant" cells, when transferred to various nutrient media, never change into rod forms.

The ability of globular cells to multiply is completely lost. The occasionally observed apparent budding is simulated by the adhesion of small subrounded cells to larger ones.

With the passage of time, the giant cells break up and the usually thinned membrane ruptures, releasing the granular content of the cell. In some cases, the giant cells undergo lysis without rupture of the membrane.

The ability to form "vesiculose" forms is common to all bacteria but, among the spore-bearing bacteria, occurs more easily in B.mycoides. Thus, this reaction can apparently be used for the identification of B.mycoides, although Imshenetskiy is doubtful of this.

On saline media, together with "vesiculose" cells, B.mycoides also gives fusiform, clavate, citriform, and other types of giant cells, which have the same structure as the globular cells. Occasionally, several of these altered cells may revert to the rod form, in a fresh nutrient medium (Fig.69).

The formation of giant bacteria forms is observed not only under the influence of salts but also under that of other factors detrimental to the development of bacteria (high and low temperature, depletion of the nutrient medium, action of bacteriophages, etc.). The atypical forms of such cells must therefore be regarded as degenerate.

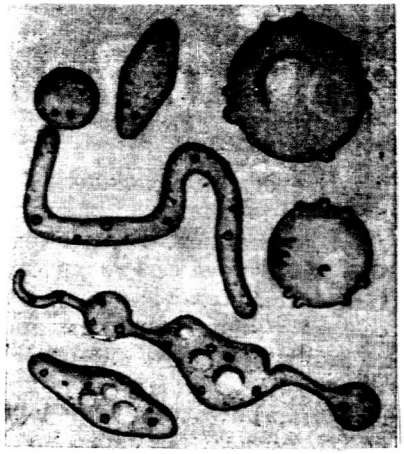

Fig.69 Involution Cells of B.Mycoides (according to Imshenetskiy)

The amorphous mass, obtained on disintegration of the formed elements and described by some authors as "symplasm", contains no viable elements. Thus, its formation must be regarded as a postmortem phenomenon.

Imshenetskiy observed the formation of "symplasm" in B.mycoides and in /118 other cultures, on ordinary and saline media. In old cultures of bacteria, agglomerates of bacteria are formed on the bottom of the test tube, resembling the packets of sarcinae or staphylococci. In saline media, their formation is accelerated.

Atypical thickened and elongated cells of B.mycoides, under the influence of phytohormones in Hottinger's medium, were obtained by Kholodnyy and Bel'tyukova (1939).

The above data thus indicate the possibility of atypical forms of B.mycoides cells. However, the phenomenon, which is basically connected with degeneration, has no relation to the so-called "developmental cycle" of bacteria.

4. Motility

In their Manual of Determinative Bacteriology, Lehmann and Neumann write that they encountered no cultures of B.mycoides with high motility. In the preparations, most rods, or a large percentage, were in the resting phase. This gives the impression that only a small number of cells have a flagellar appa-

ratus; this is also confirmed on stained preparations. Flügge (1886) and Eisenberg (1891) described B.mycoides as a nonmotile bacterium. According to data by Migula and Gottheil, B.mycoides is slightly motile. In B.mycoides, Gottheil found a peritrichous arrangement of flagella and noted that it was difficult to obtain preparations with stained flagella.

According to Marshall Word, B.mycoides has no active motility. Perlberger (1924), in studying the properties of a number of cultures of B.mycoides, described the following as nonmotile: B.mycoides Holzmüller δ and B.mycoides Gersbach; as motile: B.mycoides Holzmüller γ and B.mycoides Flügge No.3.

Stapp and Zycha (1931) described B.mycoides as a slightly motile bacterium. Kushnarev (1933), after studying several B.mycoides cultures, characterized some as motile and others as nonmotile.

Finally, it can be stated that, according to Bergey (1936), B.mycoides has slight motility and is peritrichous.

Among cultures isolated from the soils of various climatic zones, Rautenshteyn found no motile forms of B.mycoides. However, variants obtained by him under laboratory conditions did exhibit motility.

Both southern and northern variants of B.mycoides studied by us lacked true motility in the overwhelming majority of cases.

This makes it obvious that individual cultures of B.mycoides may differ /119 substantially in motility. No law on the correlation between the type of colony of B.mycoides and its motility can be established.

5. Rate of Reproduction

The rate of multiplication of cells of B.mycoides has hardly ever been studied. Some information on this question is contained in the paper by Novogrudskiy and Kononenko, who noted that various morphological forms of B.mycoides multiply at different rates on bacteriological media. On MPA, cultures of type "a" (with small cell diameter) divide fastest, cultures of type "b" (of medium thickness) divide somewhat more slowly, and the thick forms of type "c" show the slowest division.

On MPB, the forms a and b gave a pellicle after 20 hours, while the form of type c required 48 hours for this.

Differences in the rate of growth were noted only in freshly isolated cultures; after a series of transfer cultures under laboratory conditions, these differences disappeared. Thus, obviously some reorganization of the cell took place under laboratory conditions.

According to our own observations, cultures of B.mycoides with an elevated optimum temperature of growth have a tendency to more energetic reproduction. As demonstrated below, all the variants of B.mycoides inhabiting southern soils multiply at higher temperatures than those living in the soils of the northern

zones. For this reason, one can draw the general conclusion that the southern races of B.mycoides have a higher potency for reproduction under favorable conditions. This, as we will see, is of fundamental importance for the process of soil formation.

6. Spores

Lehmann and Neumann stated that the spores of B.mycoides are ovoid in shape and vary in size.

According to Bergey's Manual of Determinative Bacteriology, B.mycoides has centrally arranged spores. Their size is given as 0.8 - 1.0 μ in width and 1.4 - 2.2 μ in length. They are often arranged in the form of a chain, together with persistent residues of spore capsules. Germination of the spores is polar.

The following Table lists the findings of several authors on the size of B.mycoides spores (Table 14).

As indicated by these data, the spores of different cultures of B.mycoides evidently vary in size and shape. Findings of an ellipsoidal shape of the /121 spore predominate. The data as a whole indicate that the width of the spore usually ranges from 0.75 to 1.2 μ, and the length from 1.2 to 2.4 μ.

Certain authors note that the rod is often swollen at the site of the spore. In some cultures, the matured spore remains covered by the parent membrane for a rather long time, while in other cultures it is rapidly freed of it.

Judging from isolated observations (Rautenshteyn, Mishustin, Holzmüller) one would think that the shape and size of the spores, their formation in the cell, and their germination are not identical among the variants of B.mycoides. However, the available material is very scanty and it is difficult to make any final statements in this respect.

The process of sporulation, as indicated by the work to be presented below, is successful only under certain conditions. For example, B.mycoides is asporogenic under limited access to air. Owing to this fact, sporulation in liquid media is observed only in cases where a pellicle is formed (Stapp and Zycha).

If the bacterial mass is removed from the liquid medium and given good aeration, sporulation will occur sooner the older the culture. For example, in one experiment by Holzmüller, a two-day broth culture yielded spores within 20 hours under aerobic conditions, a six-day culture in 6 - 8 hours, and a 14-day culture in 1 hour.

In old cultures and liquid media, all preliminary stages of cell development obviously take place while sporulation often does not occur, due to an insufficiency of oxygen.

The reserve of nutrient substances in the medium exerts a major influence on the rate of sporulation.

TABLE 14

SIZE AND POSITION OF SPORES IN B.MYCOIDES

Investigator	Culture	Width (in μ)	Length (in μ)	Position of Spore	Form of Spore	Remarks
Flügge	*Bac. mycoides*				Oval	
Marshall Word	»	1.5	1.75—2.0		»	
Varlikh	»	0.6	1.3—1.8			
Gottheil	»	0.83	1.4—2.2	median		
Migula	»	0.74—0.9	1.3—1.48			Spore-bearing rod, swollen
Holzmüller	*Bac. mycoides* type α	0.8—1.12	1.5—1.8		elliptical	
	type γ	0.58—0.89	1.25—1.68			
	type δ	0.7—1.22	1.73—2.43			
Perlberger	*Bac. mycoides Flügge*	0.88—0.91	1.4—2.4			
	Bac. mycoides Holzmüller, type δ	0.9—1.0	1.3—1.5		circular or oval	
	type γ	0.9—1.0	1.3—1.5			
	Bac. mycoides Flügge № 3	1.25 1.14—1.4	1.52 1.5—1.8		circular oval	
	Bac. my oides № 4					
	Bac. mycoides Gersbach	1.02 0.76	1.5		circular obovate	
Stapp and Zycha	*Bac. mycoides*					
Kushnarev	»	1.0	1.5			
Bergey	»	0.5—1.0	0.75—2.0			
Rautenshteyn	Northern cultures *Bac. mycoides*	0.8—1.0	1.4—2.2	median		
	Southern cultures *Bac. mycoides*	0.8—0.9 1.2—1.3	1.2 1.5	close to pole		
Mishustin and Mirzoyeva	Various cultures *Bac. mycoides*	1.10—1.21		central		

To outline this proposition, let us first consider the work done by Holzmüller. He stated that sporulation sets in earlier on a poor 1% agar medium than on a rich medium. On gelatin, spore formation is slower than on agar. In all probability, this can be explained by the better diffusion of nutrients into the cell on liquefied gelatin. Certain difficulties in the access of the cells to oxygen may also be involved here.

Stapp and Zycha mentioned the important role of the composition of the medium in sporulation and specifically noted that, on protein-rich media, only half of the cells form spores while the other cells die without having sporulated.

Other authors reported similar results (Rautenshteyn, Novogrudskiy, and /122 others). Novogrudskiy reports that in soil, i.e., in a medium of poor composition, B.mycoides rapidly forms spores.

Also Pringsheim and Langer were concerned with the question of sporulation. They stated that this process begins with the utilization of the nutrient material in the medium and the accumulation of metabolites. In a colony growing on a solid nutrient medium, spore formation begins in the central portion of the colony and gradually shifts toward the periphery. At a certain stage of development, the peripheral part of the colony may still be growing while sporulation already proceeds at the center.

TABLE 15

GERMINATION OF B.MYCOIDES SPORES AND THEIR FORMATION FROM VEGETATIVE CELLS ON MEDIA OF VARIOUS COMPOSITIONS

Concentration of Agar in Medium (in %)	Time (in Days) Required for Sporulation on Media of the Following Composition			Time (in Hours) Required for Spore Germination on Media of the Following Composition		
	1/10*	1/20	1/100	1/10	1/20	1/100
1	11	13	11	28	10	96
2	11	16	16	24	77	112
3	18	16	22	64	120	228

* The media contained nutrients in proportions of 1/10, 1/20, and 1/100 of the amounts usually found in MPA.

At incipient sporulation, the long filaments constituting a B.mycoides colony often break up into short segments of 2 - 5 cells. These short chains sometimes turn toward each other at a certain angle. This phenomenon is apparently due to enlargement of the cells taking place after division of the pseudomycelium. A similar phenomenon is observed in certain algae grown on agar.

Pringsheim and Langer also found that by far not every cell will form

spores on richer media (0.5 - 1% peptone and meat extract).

On poor nutrient media (for example, with only 1/10 - 1/100 the normal content of peptone and meat extract), a spore can be found in each cell. In the latter case, the bacterial filaments do not break up so that the spores on the agar culture are arranged in orderly rows (chains).

An increase in the agar content of the medium decelerates both colony growth and sporulation (Table 15).

In the opinion of Pringsheim and Langer, sporulation is not due to the /123 growth of the bacterial cell, since the transfer of old cells to a fresh medium completely suppresses spore formation. It is hardly possible to agree with this view, when considering the highly distinct results of the Holzmüller experiments.

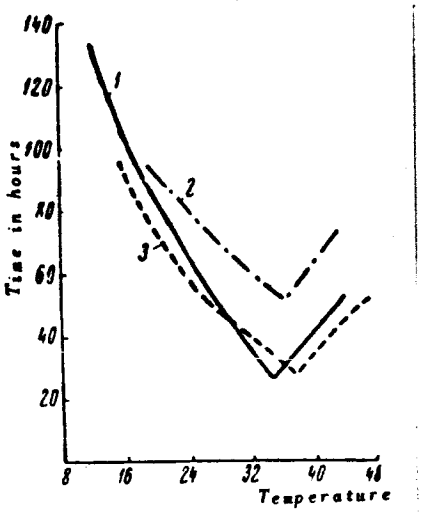

Fig.70 Rate of Spore Formation of a B.Mycoides Culture as a Function of Temperature (according to Holzmüller)
Numerals indicate:
1 - B.mycoides Flügge;
2 - B.mycoides α;
3 - B.mycoides β.

Holzmüller noted that, besides a deficiency in nutrients, insufficient moisture may also favor spore formation.

A general idea as to the effective temperature for the rate of spore formation is given in Fig.70.

All causes that produce degenerative alteration of the cells will interrupt the sporulation process, since the spores can form only on normal cells. This is explained by a number of data in the published descriptions. Thus, according to Holzmüller and own observations, proportions of NaCl high enough to cause

involution of the cells will also suppress the sporulation. It should be noted that to stop the growth of B.mycoides takes higher NaCl concentrations than to inhibit sporulation.

At a temperature close to the maximum, sporulation is stopped completely although the vegetative cells (which assume an involution form) continue to grow.

Recently, Knaysi (1945) clarified the influence of various factors on the sporulation of B.mycoides. According to his data, the metabolites (acids) retard sporulation. The useful role of oxygen consists here in oxidation of the accumulated metabolites that interfere with sporulation.

Vitamin B_1 promotes sporulation but has no substantial effect on the growth of a culture. This leads to the conclusion that the effect of the vitamin is indirect and has to do with the accelerated oxidation of acid products.

$MgSO_4$ intensifies growth, and when access to oxygen is restricted, also /124 accelerates sporulation.

Individual observations on cytological changes in the B.mycoides cell during sporulation were reported by various authors. Imshenetskiy made the most detailed study in this direction. He noted that sporulation begins with the appearance of a sporogenous zone at one end of the cell. This zone is a region of less refractile cytoplasm. The zone contains no granules, is homogeneous, and occupies 1/4 to 1/3 of the cell. The outer margin corresponds to the cell membrane, while the inner part is not sharply demarcated from the rest of the cell contents.

The outlines of the sporogenous zone then become more distinct, and its refractility begins to increase. This process of development takes 45 - 60 min and ends with the formation of a large spore.

Up to the instant of formation of the spore in the cell there may be lipoprotein granules. These have been described as "sporogenic" granules; when they merge, a "prosporangium" is formed. These granules, however, have nothing to do with actual formation of the spore. Further than that, cells rich in lipoprotein granules cannot give spores. During sporulation, the lipoprotein bodies are forced to the opposite end of the cell. Their displacement is passive. The spiral formed by the chromophilic filament and by the lipoprotein bodies only changes its location.

As it matures, the prosporangium decreases rather rapidly in size. Its shrinkage takes 15 - 20 min. The mean size of the prosporangium is 1.37 × × 1.14 μ; and the mean size of the spore is 1.25 × 0.9 μ.

Imshenetskiy correlates the shrinkage of the prosporangium with its water loss and condensation of its contents. The morphological changes can be explained only by dehydration of the contents.

On shrinkage, the spore assumes an oval shape, and its outlines become

sharp. It no longer lies so close to the cell wall (Fig.71).

The details of the change in the cell contents of B.mycoides during sporulation are described in the extensive report by Marshall Word and, in specific detail, in the book by Imshenetskiy: "Bacterial Structure".

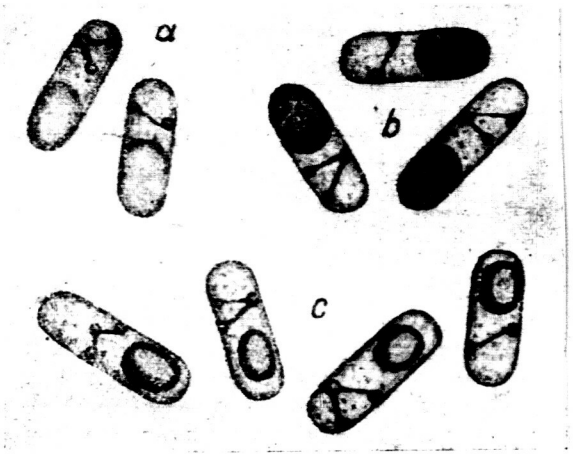

Fig.71 Formation of Spores in B.Mycoides (according to Imshenetskiy)
a - Cells with sporogenic zones; b - Cells with prospore; c - Cells with mature spore (according to Imshenetskiy)

Various authors have studied the germination of B.mycoides spores. These spores usually were found to show polar sprouting from one end or, less often, from both ends.

Holzmüller (1909) stated that this proceeds differently in different cultures of B.mycoides. He established the following types of spore germination:

1. Polar germination, in which the spore capsule stays for a long time /125 at the tip of the rod.

2. In some cases germination is equatorial. As shown in Fig.72, the germinating rod bends, divides in half at the bend, and causes two rods to sprout from the spore.

3. Still less often the spores germinate at both ends.

The ratio of these modes of germination in B.mycoides of type α is 10:2:1, and for δ it is 10:6:1. In type γ, the spores germinate equatorially, and the rods begin to grow in a radial direction. Less often germination takes place by the second of these types.

A strain of B.mycoides Flügge (culture from the Krahl Museum) almost always

yielded polar propagation, and very rarely did the spores germinate in comma form.

According to data by Gottheil and Guillermond, the germination of spores in B.mycoides is equatorial.

Some authors noted that the spore swells considerably before germination. Thus, Stapp and Zycha, 15 minutes after inoculation of B.mycoides spores into a liquid nutrient medium, found that they swell strongly but without decrease in optical refraction. The process of polar germination of the spore began within 2 - 3 hours at 28° C. The empty capsule of the spore remained for a long time at the tip of the developed bacterial filament.

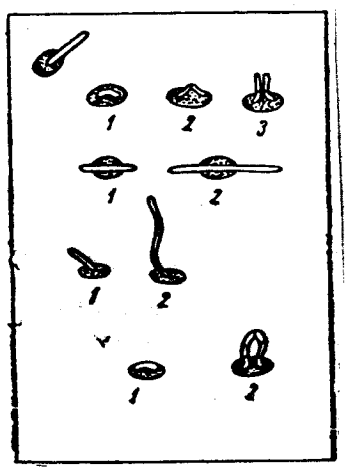

Fig.72 Germination of B.Mycoides Spores
The numerals indicate the successive stages of development of spores germinating differently (according to Holzmüller)

The conditions necessary for spore germination were investigated by a /126 number of authors. According to Holzmüller, the spores will germinate within 8 - 13 hours on ordinary MPA at optimum temperature. The specific features of the culture involved have an important effect on the rate of germination.

An addition of 0.4% of NaCl to the medium did not retard germination, while a 1% concentration of NaCl did appreciably delay it (up to 16 - 24 hours).

The concentration of the peptone and meat extract affects the rate of spore germination. A decrease in the peptone content from 1 to 0.5% and of the meat-extract content to 0.2% accelerated the germination of B.mycoides spores by 1 - 2 hours. A further decrease in the concentration of these substances retarded the spore development.

When the osmotic pressure was increased to normal on a medium depauperated in nutrient elements by adding certain amounts of NaCl, the germination of

spores was the same as on an ordinary nutrient medium. This definitely indicates the important role of osmotic pressure in spore germination. It is interesting to note that, in distilled water and physiological saline solution, the spores of B.mycoides did not germinate at all. This indicates that the nutrient material has a definite significance for spore development.

On solid nutrient media, not only the concentration of the nutrient but also that of the agar has a major influence on the germination of B.mycoides spores. In experiments by Pringsheim and Langer, agar media containing 1/100 and 1/10 of the normal concentrations of nutrients and various percentages of agar were used. It is obvious from the results (Table 15) that the presence of a certain nutrient reserve has a strong effect on the energy of spore germination. Similarly, an increase in the percentage of agar occasionally has an influence. For example, in media containing 3% agar, spore development is strongly retarded. On a medium containing 4 - 6% agar, most of the spores did not /127 germinate. In the spores that did germinate, the growth of the short bacterial filaments often stopped again.

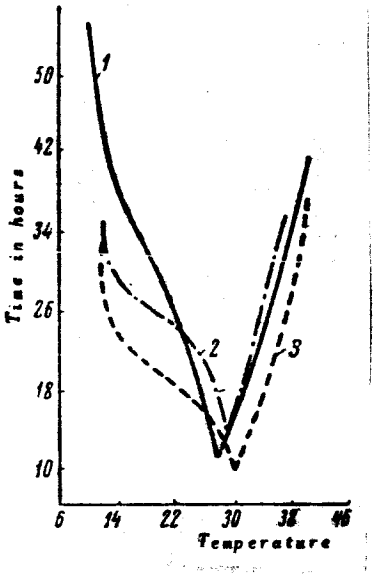

Fig.73 Effective Temperature for Germination of B.Mycoides Spores (according to Holzmüller)
Numerals denote:
1 - B.mycoides Flügge;
2 - B.mycoides α;
3 - B.mycoides β.

In comparing a number of published data, we find that spores of B.mycoides germinate considerably faster in liquid media than on MPA.

According to Imshenetskiy, the most prolonged stage in germination of a spore is that of swelling, during which the size of the spore increases $1\frac{1}{2}$ to 2 times. The process of abstriction from the rod is rather fast.

In general the entire process of swelling of the spore is accompanied by almost the same morphological changes as the formation of the spore, but in the reverse order.

Simultaneously with these morphological changes, the enzymes of the spore are activated, the capsule of the spore breaks open, and the rod sprouts through the hole.

Holzmüller concluded that B.mycoides spores can germinate in a fairly wide temperature range. For the cultures studied by him, he indicates the principal temperatures for spore germination: minimum at 10 - 12° C, optimum at 29 - 31° C, and maximum at 39 - 41° C (Fig.73).

Among the individual ecological races of B.mycoides the above temperature points presumably will vary somewhat.

Spore germination evidently does not require the presence of oxygen. According to Holzmüller, the spores of B.mycoides germinate just as well under anaerobic conditions as in the presence of oxygen.

The experiments of this author also indicate that diffuse light has no appreciable effect on the germination of B.mycoides spores. However, an exposure of the spores to direct light, though only for a short time, did retard their growth. A protracted exposure to sunlight impaired the subsequent growth of colonies of some B.mycoides cultures while others, after germination of the spores, did not develop further since the cells disintegrated after leaving the spores. In many B.mycoides cultures, the spores did not germinate at all /128 after exposure to sunlight.

TABLE 16

DEATH OF SPORES OF VARIOUS CULTURES OF B.MYCOIDES AT 100° C
(Mean Data of Nine Experiments)

Heating Time, in Min	Number of Viable Spores Remaining after Various Heating Periods		
	Igarka Culture	Moscow Culture	Central Asian Culture
0	226.000	284.000	255.000
3	851	2.065	6.442
6	265	183	2.826
9	103	130	990
12	82	50	656
24	40	46	139
45	0	2	6
48	0	0	3

It may be stated that spores of B.mycoides do not possess great heat resistance. Thus, Lamanna (1942) showed that spores of individual cultures of

B.mycoides die within 2 min at 95° C.

In our own work, we found excellent high-temperature resistance of spores in cultures that develop at elevated temperatures. Hence, a greater heat resistance should be expected in the southern cultures of B.mycoides. Rogacheva, in studying the thermostability of spores of the various geographical tribes of B.mycoides taken from our collection, came to the same conclusion. The spores of southern cultures of B.mycoides were more thermostable than spores of the northern forms (Table 16).

According to Fred (1938), spores of B.mycoides will die within 15 min at 100° C. According to Meyer, heating the spores in boiling water kills them within 9 - 10 min.

According to data by Novogrudskiy, spores of the thin forms of B.mycoides are most resistant to heat. After heating for 30 min at 90° C, spores of two thin-cell cultures out of three taken for the experiment were not completely destroyed. Spores of forms with moderate-size cells died within 30 min at 90° C, while spores of thick-cell forms did not even tolerate heating at 90° C for 10 - 20 min.

Virtanen showed that spores of B.mycoides strains available to him died /129 within 60 min at 90° C and within 30 min at 100° C.

Magoon (1926), in speaking of the resistance of B.mycoides spores to elevated temperatures, mentioned that this property may vary within the very same culture, depending on the age of the spores, and the ambient temperature and humidity.

To answer the general question as to the causes of heat resistance of spores in general, Virtanen (1933) investigated B.mycoides. He stated that the resistance of bacterial spores to high temperature must be explained either by the possibility that the spores have a capsule protecting them from external influences or by certain properties of the cell contents.

Dehydration of the cell protoplasmic matter, which would mean that the spores are more hygroscopic than the vegetative cells, has often been mentioned (Cramer). This conclusion, however, is based on analogies with the spores of molds since, before Cramer's time, no direct determinations of the properties of bacterial spores had ever been made.

The literature on enzymes, among other things, mentions that bacterial spores have active ferments (Effront, Ruehle).

The chemical composition of spores and vegetative cells of B.mycoides, as investigated by Virtanen, indicated no major difference between them. A certain difference existed only in glycogen content. However, the ferment system in the spores proved somewhat less active than in the vegetative cells. It is the low fermentative activity of the spores that in Virtanen's opinion explains their heat resistance.

Of course, the view of Virtanen, insofar as it relates to the hydrous

properties of bacterial spores, requires confirmation since the entire picture of the morphological changes of the cell during maturation, and particularly the shrinkage of the prospore, are evidence of protoplasm dehydration in the formed spore.

The data by Friedman and Henry, which deserve attention, indicate that the varying resistance of bacterial spores to elevated temperatures is explained by the ratio of bound to free moisture. In the heat-resistant forms of bacteria, the spores are richer in bound moisture (Table 17). As shown by these data, the low heat resistance of B.mycoides spores can be explained by the presence of a considerable quantity of free moisture. In bacterial species yielding spores more resistant to elevated temperatures (B.subtilis and B.megatherium), the spores are richer in bound moisture.

TABLE 17 /130

BOUND AND FREE WATER IN SPORES OF VARIOUS BACTERIA

	B.Subtilis		B.Megatherium		B.Mycoides	
	Bound Water	Free Water	Bound Water	Free Water	Bound Water	Free Water
Percent of water in bacterial mass	69.0	3.4	62.6	4.6	58.7	11.6

Figure 74 is an electron micrograph of the spores of one of our cultures, made by A.Ye.Kriss and Ye.A.Rukina of the staff of the Institute of Microbiology, USSR Academy of Sciences.

Fig.74 Electron Micrograph of Spores of B.Mycoides (Taken by Kriss and Rukina)

7. Asporogenic Strains of B.Mycoides

As noted above, cultures of B.mycoides in many cases are unable to form spores. Thus, it was found as far back as Holzmüller's time that the involution cells of B.mycoides did not form spores. This observation was later confirmed by a number of investigators (Lewis, Mishustin, and others). We already /131 mentioned the fact that, by changing the nutrient regime, the sporulating tendency of cultures of B.mycoides may be temporarily suppressed.

Rautenshteyn (1937) stated that cultures of B.mycoides do not form spores on protein media. This change in the properties of the culture may be regarded only as a prolonged modification, since on ordinary media sporulation was again resumed.

Den Dooren de Jong (1923), in cultivating B.mycoides on media with a higher peptone and sugar content, also observed a complete suppression of sporulation. On transfer culturing to ordinary media, however, sporulation resumed. Asporogenic forms with transmitted characteristics were obtained by the above author on transfer to solid media of B.mycoides cultures grown on 1% peptone water. Not all cultures of B.mycoides yielded asporogenic forms when so treated. The obtained mutants differed from the ordinary forms in the greater transparency of their colonies. This trait was fairly constant.

Some investigators succeeded by chance, or by exerting certain influences on a B.mycoides culture, in obtaining asporogenic forms. Nadson and Adamovich described such a culture in 1910. Similar forms were obtained by Nyberg and by Oesterle (1927, 1929), and also by Kushnarev (1932).

Borodulina (1933) noted the formation of asporogenic variants of B.mycoides under the effect of metabolic products of actinomycetes on a culture of this microorganism. On mineral-peptone agar, the asporogenic form yielded smooth hyaline colonies, with branching within the agar.

8. Development of B.Mycoides on MPA and Direction of Curvature of Bacterial Filaments in its Colonies

The formation of colonies of B.mycoides was discussed in detail in the last Chapter. In this Section, we will discuss only the question of the curvature of the bacterial filaments in a colony of B.mycoides.

As already mentioned, most soil-inhabiting variants of B.mycoides, on agar media, will form colonies where most of the bacterial threads are sinistral (turning counterclockwise). Less often we find "isomeric" forms with dextral rotation.

This phenomenon was first reported by Neisser and was later studied in detail by Pringsheim and Langer, and by Gersbach.

This led to postulating the existence of some sort of stress in an agar /132 medium, causing a deflection of the growing strands to the right or to the left.

Gersbach tried rotating solidified agar counterclockwise in one dish and clockwise in another, so as to establish different senses of the vectorial forces in the medium. However, this had no effect on the character of colony growth.

A similar experiment, in a different technical mode, likewise failed to yield an affirmative answer. A small Petri dish was rotated in one sense, during solidification of the agar. This dish was then placed in another larger dish, and the space between the walls of the two dishes was also filled with agar, while rotating both dishes in the opposite sense.

A culture of B.mycoides was inoculated into the center of the small Petri dish; as this culture gradually grew, it spread to the agar in the larger dish. The sense of rotation of the strands on the agar in both dishes was identical.

If an imprint of a fully grown colony with sinistral whorls was made on a free agar surface, a mirror image of the first culture was obtained. However, on keeping such a replica in the incubator, the subsequent growth of the strands continued in the original sense of rotation of the given culture, i.e., the B.mycoides culture did not become dextral but remained sinistral.

It is of interest to note that the strands of B.mycoides retain their sense of rotation relative to the surface of the medium. If a pour plate of MPA in Petri dishes is so inoculated as to cause a colony of the sinistral form of B.mycoides to develop on the lower surface of the medium next to the bottom of the dish, then the strands in this portion will likewise rotate counterclockwise, relative to the agar surface. When the dish is viewed from above, the impression is gained that the surface colony has strands directed in one sense and the bottom colony, strands in the other sense.

Gersbach's attempt to change the sense of rotation of the bacterial strands by a suitable choice of nutrient media was unsuccessful. He also found that gravity had no appreciable effect on this phenomenon.

Morphologically, he found no difference in the dextral and sinistral strains of B.mycoides. Similarly, he discovered no substantial differences in the vigor of colony growth or in the resistance of the spores to adverse influences. Serological reactions likewise confirmed the identity of the dextral and sinistral forms of B.mycoides.

Gauze studied the features of the mirror-image forms of B.mycoides. These cultures were found to be identical with the ordinary sinistral forms in /133 their main properties, but had entirely different growth-temperature relationships. The rate of growth of the sinistral variants increased exponentially with rising temperature, while the dextral variants were damaged by heat causing their temperature-growth curve to assume a domed depressed form.

We believe that Gauze's conclusion should not be generalized, since he was dealing only with a limited number of B.mycoides cultures.

In our own work, we encountered sinistral forms which also showed the phenomenon of "thermotic damage". The observations of Alpatov and Nastyukova are

very interesting. They showed that the growth of the dextral forms of B.mycoides is more inhibited by dextrorotary quinacrine hydrochloride, and that of the sinistral forms by levorotary quinacrine hydrochloride.

Up to now, no satisfactory explanation has been found for the rotation of the bacterial strands of B.mycoides on agar media in one sense or the other. Pringsheim and Langer advanced the following hypotheses:

1. It may be assumed that the curvature of the filament is due to the unequal activity of the flagella on both sides of the filament. However, many cultures of B.mycoides have no flagella, and yet their filaments on agar do turn in some sense. Moreover, the adult cells of B.mycoides lose their flagella, but the curvature still persists in the relatively old part of the bacterial strand.

2. By analogy to the higher plants, it may be supposed that one side of the bacterial cell grows more rapidly than the other. In this case, however, the end of the bacterial filament should be curved, which is not the case.

3. It may be postulated that the metabolites given off by the cells themselves act to bend the bacterial filaments. The filaments are evidently negatively chemotropic relative to their own metabolites. This view is confirmed by the picture created on encounter of branches of two adjacent colonies. Not only will the two meeting ends suffer a certain depression, but they often turn sharply aside. A similar deflection, not characteristic for the strands of a given culture of B.mycoides, may be obtained by placing some toxic substance in the path of the growing colonies.

It seems that adoption of the latter hypothesis would mean that the metabolites are not uniformly distributed on both sides of the cell. This would then be the reason for the fact that, in some cultures, the rotation is dextral and in others sinistral.

As is obvious from all of the above data, nothing reliable has been learned of the determining factors for the rotation of bacterial strands in a B.mycoides colony. /134 Numerous arguments also speak in favor of this phenomenon, on the whole, being connected with the growth characteristics of B.mycoides cells on the surface of a solid nutrient medium. We believe that curvature of a bacterial filament, if its growth is intercalary, is possible only if one side of the filament cells, for some reason, grows somewhat faster than the other. Microscopic examination of the colony edge readily shows that the cells are extensively curved only in certain segments of the filament. Apparently, these segments represent centers where the strong curving tendency inherent to all cells of the filament is especially pronounced. This process is easily visualized by assuming that the tip of the filament, in the medium, meets a purely mechanical obstacle to its advance, somewhere along its path. Here, the filament begins to turn aside; this turn is especially distinct at any point where there is the chance of a minor sideslip.

The capability of various forms of B.mycoides to form colonies of a definite structure (dextral or sinistral) is transmitted by heredity, and no author has ever succeeded in reproducing it under experimental conditions.

In addition, it may also be concluded that the composition of the solid nutrient medium has a substantial influence on the filament curvature in a B.mycoides colony. For example, Pringsheim and Langer succeeded in obtaining such colonies on a low-agar medium, with quasi-radial strands. With increasing agar content in the same medium, the filament curvature increases in sharpness. The aqueous pellicle on the surface of the solid medium becomes more tenuous as the agar concentration increases. This, of course, increases the mechanical resistance to the advance of the growing filament in a radial direction. The sliding of the upper cells is impeded, and the filament begins to turn to one or the other side. The sense of the turn is determined by the features of cell development, discussed above.

In our opinion, the degree of rotation of the filaments in a B.mycoides colony is determined by the unequal growth of the cell sides as well as by the properties of the water film formed on the surface of the agar medium. Various observations, to be discussed later, indicate that the absolute dimensions /135 of the water film are not the only factor of importance, and that the existence of some vectorial forces in that film, due to components of the nutrient medium, also plays a role. The value of the former factor is simply established by drying the medium in the Petri dish. This always has a marked effect on the stresses of the coils and on the sharpness of the turns. The role of the orienting forces of the water film is proved by the fact that, in media of the same agar content but of different composition, the type of coils formed by the B.mycoides strand differs greatly. For example, on potato agar the colonies always contain strongly curved filaments while, on an agar medium with 0.1% peptone, the curvature is considerably less or the strands may actually assume a radial direction. The traits of the particular strain also have a major influence on the character of the deflections.

The above phenomenon can be explained only by assuming that certain vectorial forces, produced by the components of the nutrient medium, must exist in the water film. Check tests showed that the optical properties of the substances contained in the medium play no substantial part in this phenomenon. For example, on peptone agar with 0.1% peptone and various admixtures of carbohydrates (levulose, dextrose, saccharose, etc.), different cultures of B.mycoides will form colonies with coils of almost identical character on these media that differ only in the type of sugar added.

On the basis of these propositions, it could be expected that the presence, in the water film, of forces counteracting the natural curvature of the filaments will cause these to grow radially or even to curve in the opposite sense. Of course, in the latter case, the energy of the orienting forces of the medium must exceed the bending force produced by the uneven development of the bacterial cell. In other words, it was theoretically to be expected that, on certain media, the sinistral forms of B.mycoides could be transformed into their mirror images. This was indeed observed by us on certain media, when testing their suitability for growing B.mycoides. Thus, on a medium containing 0.1% peptone, 0.1% K_2HPO_4 and 1.5% agar (Arkhangel'sk Plant), certain cultures of B.mycoides showed a distinct sinistral rotation. When the agar content was increased to 2.5%, the strands became randomly (radially) oriented in some cultures, while in others they showed distinct dextral coils. In some cultures of

B.mycoides, an increase in the percentage of agar had no effect on the character of the colonies. This can be explained by a difference in the relationship /136 of the forces responsible for the filament curvature. If the curvature of the cell is completely suppressed by forces existing in the medium, then the structure of the colony will be modified; a sinistral form, for example, may be transformed into a dextral form. However, if the unequal growth of the cell produces a force exceeding the other forces in the medium, then the type of colony will remain unchanged. In the intermediate situation, the bacterial strands assume a radial direction.

In our work, we compared the development of 75 cultures of B.mycoides isolated from various soils, on three media: MPA, peptone agar, and potato agar. Each of these media were prepared in two versions: the first containing 1.5% agar and the second, 2.5%.

In preparing MPA, we used the dry medium developed by Engineer Nevizhin, which is very rich in amino acids. B.mycoides give denser colonies with less sharply curved filaments on this agarized Nevizhin medium than on ordinary MPA.

The peptone agar was prepared from tap water with admixture of 0.1% peptone and 0.1% K_2HPO_4.

The potato agar was prepared by boiling 300 gm potato in 1 liter water and adding the required amount of agar to the filtered medium.

The simultaneously prepared media were poured into Petri dishes at about 43° C. After solidification of the agar, stab cultures were started on the medium.

On potato with 1.5% agar, the great majority of B.mycoides cultures, isolated from various soils, exhibited sinistral forms. Only a single culture, isolated from Moscow soil, gave an indeterminate direction of the coils.

An increase in the agar content of the potato medium disoriented the direction of the bacterial strands in several cases. One culture (Igarka No.8) even formed a dextral colony.

On peptone with 1.5% agar, a considerable number of B.mycoides cultures had an indeterminate orientation, and the forms that still retained their sinistral character were appreciably less curved. In general, strongly curved strands were formed on potato-agar media.

The addition of 2.5% agar to the peptone medium led to a mass reorganization in the colony structure. In most of the cultures, the strands assumed an indeterminate orientation, and in a considerable number of cases sinistral forms changed to dextral. Of course, such a reorganization was not hereditary and, /137 on transfer to the usual medium, all these altered cultures of B.mycoides began to form colonies of their own inherent type.

The random orientation of strands of B.mycoides colonies was observed in a considerable number of cultures on MPA. An increase in the agar content also increased the number of such forms. Only occasional cultures were dextral.

Table 18 gives a summary of the results of this experiment.

TABLE 18

DIRECTION OF BACTERIAL STRANDS IN B.MYCOIDES COLONIES ON VARIOUS MEDIA
(Percent of Cultures, Related to their Total Number, Giving Particular Rotation of the Strands)

Sense of Strands in Culture	MPA		Peptone Agar		Potato Agar	
	1.5	2.5	1.5	2.5	1.5	2.5
Igarka						
Sinistral	60	50	50	—	100	90
Dextral	—	—	—	40	—	10
Indeterminate	40	50	50	60	—	—
Moscow						
Sinistral	58	48	53	11	—	79
Dextral	—	—	—	36	—	—
Indeterminate	42	52	47	53	—	21
Sumy						
Sinistral	25	25	75	75	100	50
Dextral	—	25	—	—	—	—
Indeterminate	75	50	25	25	—	50
Samarkand						
Sinistral	81	62	38	19	100	75
Dextral	—	—	—	19	—	—
Indeterminate	19	38	62	62	—	5
Frunze						
Sinistral	60	21	13	7	100	93
Dextral	7	13	21	53	—	—
Indeterminate	32	66	66	40	—	7
Golodno Step'						
Sinistral	64	64	73	73	8	64
Dextral	9	9	9	18	—	—
Indeterminate	27	27	18	9	18	36

The Table again shows that there was a marked reorganization of colony /138 structure on the peptone agar, especially when the agar content was increased to 2.5%.

Figure 75 indicates the great difference in orientation of the strands on various media.

All these facts clearly demonstrate the role of the surface film in the orientation of bacterial strands. It seems that, without the above assumptions, it is impossible to explain the different senses of the strands on media of different composition but equal agar content.

Our opinion as to the role of the water film can also be confirmed by a simple experiment, which consists in drying the agar surface. Preparing a pour plate of peptone with 2% agar in a Petri dish and drying it slightly in a drying

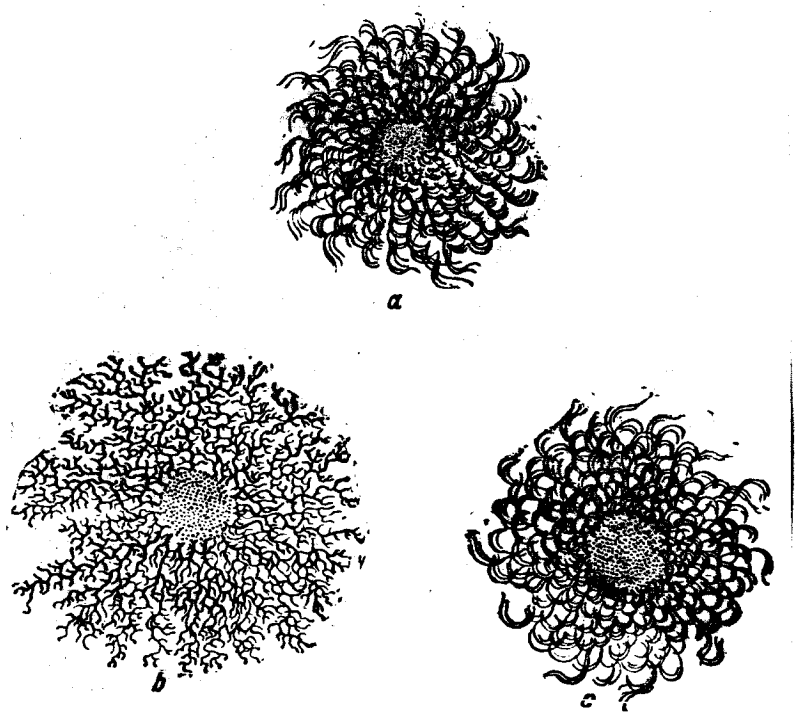

Fig.75 Orientation of B.Mycoides Strands on Various Media
a - Colony on MPA; b - Colony on peptone agar (1% agar);
c - Colony on peptone agar (2% agar)

chamber, will induce a tendency to form mirror-image coils in a number of /139
B.mycoides cultures. Table 19 gives the results of such an experiment. We used three cultures of B.mycoides which, on 1.5% potato agar, gave sinistral coils. Two of them, on 2% peptone agar, gave a radial growth; however, on the same medium, when slightly dried, their colonies were dextral. One culture in this experiment still formed sinistral colonies on all media.

In our opinion, this can be explained by the fact that, as a rule, forces that counteract the normal curvature of the filaments appear in the surface film of a peptone medium. Their action becomes more pronounced as the thickness of the water film is decreased by drying. Of course, the action of increased amounts of agar is due to a similar cause. For example, pouring 2.5% agar media hot into Petri dishes favors retention of the usual sense of rotation of the strands for the culture in question. This fact is entirely understandable since, in this case, a relatively heavy water film is formed on the surface of the medium.

We have already noted that not only the thickness of the water film on the

surface of the medium but also the composition of the solutes affect the orientation of the strands. In fact, on 2.5% potato agar our cultures gave entirely normal colonies, while on 1.5% peptone agar (which undoubtedly has a thicker water film), most of the cultures were disoriented. We are unable to define the forces arising on the agar surface. These must be studied by physical chemists, but their presence is proved by the mode of development of the microorganisms.

TABLE 19

EFFECT OF DRYING OF AGAR ON THE ORIENTATION OF BACTERIAL STRANDS IN B.MYCOIDES COLONY

Cultures	Agar Medium, Not Dried	Agar Medium, Dried
Moscow, No.20	i	d
Same, No.3	s	s
Igarka, No.8	i	d

Symbols used:

i - Orientation of strands, indeterminate;
s - Orientation of strands, sinistral;
d - Orientation of strands, dextral

The following experiment also indicates the decisive effect of the /140 solutes, contained in the medium, on the orientation of the structural elements of the colony. The use of differing amounts of peptone in a medium with the same agar percentage will affect the sense of growth of the bacterial strands.

Table 20 gives the results of one of these experiments, requiring no special explanation. Naturally, each culture, for obvious reasons, reacts in a specific manner to the addition of peptone.

TABLE 20

EFFECT OF PEPTONE CONCENTRATION ON THE SENSE OF ROTATION OF BACTERIAL STRANDS IN A B.MYCOIDES COLONY

Culture	1.5% Agar with Peptone Concentration:			3% Agar with Peptone Concentration:		
	0.1%	0.5%	1.0%	0.1%	0.5%	1.0%
Igarka, No.11	s	i	i	d	d	i
Moscow, No.4	s	i	i	i	i	i

These data disclose some of the causes affecting the topography of B.mycoides colonies. They show that the tendency of B.mycoides cultures to turn in a certain sense is inherited and constant on a given medium. However, by changing the composition of the nutrient substrate, a new orientation of the strands in the colony can be obtained.

We emphasize again that all forms of B.mycoides, isolated from soils of the USSR, generally yielded sinistral colonies on MPA. We were able to isolate dextral cultures only from the soils of mountain regions (Urals, Causasus).

9. Growth of B.Mycoides on Agarized and other Bacteriological Media

On slant agar, B.mycoides yields grayish-white glistening pellicles with extremely abundant rhizoid shoots which, after a certain length of time, cover the entire agar surface. In the central part of the streak, a thicker accumulation of bacterial mass is often formed. In time, filamentous bacterial strands sometimes penetrate into the agar.

In certain B.mycoides cultures, the character of the streak varies slightly and, occasionally, even greatly.

One of the forms of B.mycoides (B.mycoides α citreus), isolated by Neumann, yielded lemon-yellow colonies on agar. A similar form, but with less marked /141 pigmentation, was isolated by us from soils of the tundra near Igarka. This form differs somewhat in colony structure from the typical B.mycoides, and its yellowish tint persisted for three years of transfer culturing. Later, it began to give colorless colonies on agar, although the structural traits persisted. Perhaps, this was due to the inferior media used in the laboratory during the war.

Agar stab cultures caused the development, in the interior of the medium, of numerous thin shoots running parallel to each other and perpendicular to the direction of the stab. Their length varied. Colonies of B.mycoides characteristic for MPA developed on the surface of the medium.

On meat-peptone broth, the growth differed somewhat in the individual cultures of B.mycoides. Usually, a pellicle, that readily sank to the bottom of the test tube, developed on the surface of the medium. The interior of the broth remained almost transparent when rugose forms were cultured. The smooth forms caused considerable clouding.

In the course of time, a sediment forms on the bottom of the test tube. In the rugose forms of B.mycoides, the sediment is somewhat difficult to break up. The sediment in the smooth forms is easily emulsified.

MPB becomes alkaline on progressive development of B.mycoides, due to the formation of ammonia from the peptone.

Generally, no indole is formed. Occasionally, traces will be encountered. Similarly, there is no formation of hydrogen sulfide. Nadson, however, stated that cultures of B.mycoides, isolated from mud, will generate hydrogen sulfide.

It may be noted that the pellicle of B.mycoides consists of long cells. No spores are formed in the sediment, evidently because of the lack of oxygen.

On a gelatin medium the young colony consists of barely perceptible webs of bacterial filaments. Later, after 1 - 2 days, the colony increases in size and starts resembling a white dot from whose periphery radiate hyphoid shoots consisting of a large number of cells. The central portion of the colony is formed by the dense intertwining of hyphoid formations. The colony is white or has a grayish-white tinge.

Under the microscope at low magnification, the structure of the colonies looks like that on MPA.

Gradually, with progressing development of the colony, the gelatin begins to liquefy. When the gelatin is inoculated by a stab, numerous thin fibrils /142 start radiating from the streak within a few days and penetrate into the gelatin in a thick mass.

Liquefaction of the gelatin, forming a crater-like depression, begins later. The pellicle floating on the surface of the medium may sink but is immediately replaced by another. Cultures with pellicles that had sunk to the bottom as often as three times have been observed (Lehmann and Neumann).

According to Eisenberg and Migula, a pellicle that sank may rise again to the surface of the nutrient, after a certain time.

On potato, B.mycoides gives an uncharacteristic growth, resembling that of B.subtilis. The pellicle has a whitish dull lustre, changing to yellow in an old culture.

The bacterial pellicle may vary from homogeneous to granular in the various forms of B.mycoides.

According to Bergey, a brownish tinge appears in a B.mycoides culture on potato (see also Table 2).

Blood serum is not liquefied.

On milk, most cultures of B.mycoides produce coagulation; the resultant coagulum later starts to peptonize.

Nyberg (1929) and Lewis noted that the behavior of B.mycoides to milk depends on its grade. If market-grade milk is used for the experiment, it usually coagulates after sterilization and inoculation with the B.mycoides culture. No acid formation is noted in this case. The peptonization of the coagulum begins in about 10 days. If fresher milk is used, it will be peptonized under the influence of B.mycoides, without coagulating. According to our observations, the variants of B.mycoides show no substantial difference in their growth on milk.

10. Relation to Oxygen and other Chemical and Physical Factors

Relation to oxygen. B.mycoides is a facultative aerobe, although several authors consider that it should be classified as an obligate aerobe.

According to Holzmüller, when grown on MPA without access to oxygen, the bacterium may develop satisfactorily but the habitus of its colonies will differ somewhat. The colonies will be more even, with less plumose edges. Growth under anaerobic conditions in general is slower than with access to air.

Under anaerobic conditions, B.mycoides does not sporulate for a long time. If, however, oxygen is given access to the developing culture, sporulation /143 sets in rapidly. This demonstrates that the stages preceding sporulation develop better under anaerobic conditions and that a brief aeration is sufficient for the rods to form spores.

In his day Matzuschita noted that an oxygen deficiency caused retardation of sporulation in facultative aerobes.

Relation to temperature. Various authors give widely differing cardinal temperature points for B.mycoides. Thus, according to Bergey, the optimum temperature is near 30° C, according to Blau, near 30 - 35° C, according to Holzmüller, near 28 - 32°C, according to Stapp and Zycha, near 25° C, while according to Pringsheim, the optimum temperature is room temperature.

Marshall Word, in studying a culture of B.mycoides isolated from water of the Thames River, found that its minimum temperature was near 5 - 6° C, the optimum near 25 - 28° C, and the maximum near 39 - 40° C. Rautenshteyn noted that, in various cultures of B.mycoides, the position of the optimum temperature point may vary over a range of 25 - 31° C.

In our own work we discovered the causes of the different temperature ratios of individual cultures of B.mycoides. The individual traits of the given culture as well as the existence in nature of ecological races of B.mycoides (see below) play an important role here.

In speaking of the optimum temperature for B.mycoides growth, Holzmüller noted that the optimum conditions for growth and formation of shoots (strands) in a colony are not the same. More distinct strands are formed at a temperature below the optimum growth point. At temperatures above the optimum, the strands begin to be strongly twisted. At temperatures close to the maximum, the colony formed consists of a continuous bacterial mass, with weak shoots along the periphery.

A rise in temperature above the optimum has an adverse effect on the cell shape. At 35° C, degenerate cells may be found in most northern cultures. In southern cultures, they appear at a higher temperature.

In general, the temperature interval is widest for germination of spores; it becomes somewhat narrower for growth of bacteria and narrowest for spore formation. The latter fact is explained by the general failure of involution cells to form spores and by the degeneration of cells at a high temperature,

before onset of sporulation.

The spores are able to germinate above the maximum temperature of culture growth, but the cells formed disintegrate rather fast, often first passing /144 through the stage of involution forms. The cell wall undergoes lysis, and the contents agglomerate into a glistening granule. Such nucleoli then gradually dissolve.

It should be noted that southern cultures, which have a higher temperature range of development, pass through their life cycle more rapidly (at the optimum temperature); thus, their sporulation sets in earlier.

Influence of light. Diffuse light, like darkness, has no effect on the growth of a B.mycoides culture. Exposure of the spores to direct light, for even a short time, inhibits their germination. Protracted exposure to light leads to subsequent impairment of the colony growth. In this case, the spores will germinate but will not develop further, since their cells disintegrate. Some of the spores will not germinate at all after irradiation (Holzmüller, Marshall Word).

Influence of acidity of the medium. B.mycoides can grow in a rather wide range of pH values. The minimum is near pH 5.0, the optimum near 7.4, and the maximum above 8.8.

In our experiments, we tested various cultures of B.mycoides for their behavior to the acidity of the medium. The collection included both southern and northern cultures, isolated from soils of varying acidity. It could be assumed that we had to do with a certain adaptation to the environment. Nevertheless, we found no substantial differences, even between the smooth and rugose forms.

11. Pathogenicity

B.mycoides is a typical saprophytic microorganism. Nevertheless, when injected into mice, together with an acid (lactic or formic), it causes death (Much, 1921).

The culture of B.mycoides isolated by Much, when added to a broth culture of staphylococci, clarified the medium. A medium, inoculated in this manner, showed greater pathogenicity than the staphylococcus itself.

12. Conversion of Chemical Substances

Investigators of B.mycoides have repeatedly noted the differing behavior of individual cultures to carbon sources.

As noted already in 1909 by Holzmüller, B.mycoides grown on carbon-containing media will form acid without producing gas. His B.mycoides culture fer- /145 mented cane sugar but did not ferment lactose (or fermented it only slightly).

Virtanen and Pulkki (1933) found that B.mycoides mostly converts carbo-

hydrates into lactic acid (82.6%). Acetic acid (4.0%) and alcohol (0.9%) were noted as by-products.

According to Perlberger (1924), most cultures of B.mycoides ferment levulose, dextrose, maltose, raffinose, and arbutin.

Some cultures ferment saccharose, mannose, galactose, amygdalin and salicin.

Lewis (1932) who thoroughly studied a number of widely differing variants of B.mycoides, including several experimentally produced subspecies, came to the conclusion that the enzymatic power of these variants was quite similar. All of them fermented the following sugars under formation of acid: glucose, levulose, saccharose, maltose, dextrin, and salicin. They did not ferment lactose, mannite, dulcite, amygdalin, arbutin, and xylose. Some variants, obtained from daughter colonies, had a pronounced capability to ferment lactose.

Judging from the data by Kushnarev (1933), which in our opinion still require confirmation, not all cultures of B.mycoides ferment glucose and maltose. In individual cases, the fermentation of galactose and lactose was noted.

Novogrudskiy and Kononenko (1935) report that cells of a culture of B.mycoides at different depths behave differently toward sugars. However, their data contain some obscure factors. For example, they reported that forms with thick cells ferment disaccharides but do not attack glucose.

The statements by Rautenshteyn (1937) seem to indicate that a change in experimental conditions may greatly alter the relation of B.mycoides to sugars.

Table 21 is a summary of data by various authors on the relation of B.mycoides to the principal carbon sources.

The Table clearly indicates that individual cultures of B.mycoides differ in their behavior toward saccharose, galactose, and raffinose. The most reliable data point toward the assumption that glucose and maltose are fermented /146 by all cultures of B.mycoides.

It may be added to this information that, according to data by Lewis, the dextral forms of B.mycoides ferment sugars more slowly than the sinistral forms. It is true that the sinistral forms comprise some low-activity forms, but no dextral forms that vigorously ferment sugars were ever encountered.

Glinka-Chernorutskaya (1930) reported the capability of B.mycoides to split arbutin into quinhydrone and glucose. She was not, however, able to isolate arbutase by the methods generally useful for the isolation of ectoenzymes.

Since the literature contains differing data on the relation of individual cultures of B.mycoides to carbon sources, we became interested in comparing our cultures of this bacterium with respect to this particular property.

Our selection included rugose (ordinary) forms encountered mainly in northern soils, forms of intermediate type which are abundant in the northern /147 chernozems, variants of B.mycoides with strictly oriented strands occurring in

TABLE 21

FERMENTATION OF CARBON-CONTAINING COMPOUNDS BY VARIOUS B.MYCOIDES CULTURES

Author	Culture	Glucose	Galactose	Saccharose	Maltose	Lactose	Raffinose	Arabinose	Mannite
Perlberger . .	*Bac. mycoides* δ	+	−	−	+	−	+	−	−
	» » γ	+	−	−	+	−	+	−	−
	» » № 3	+	−	−	+	−	+	−	−
	» » № 4	+	−	−	+	−	+	−	−
	Bac. mycoides Gersbach	+	+	+	+	−	+	−	−
Lewis . . .	Various cultures	+	+	+	−				−
Kushnarev . .	Various cultures	+	−	+	−	−			
		Not all	As a rule	In most cases	In most cases	In most cases			
Novogrudskiy .	Thin forms	+	+	+	+	−	−	−	−
	Medium »	+	+	+	+	−	−	−	−
	Thick »	+	−	+	+	−	−	−	−
		or −		or −	or −				

Note. The plus sign means that acid is formed; the minus sign, that no acid is formed.

soils of the southern belt, and, finally, smooth forms which are rather often encountered in gray soils.

About 90 cultures of B.mycoides were selected for this study. Their behavior toward various carbon sources was determined on a liquid medium (1% peptone, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.01% $CaCl_2$, 0.5% NaCl, traces of $FeSO_4$) containing 1.0% of the test substance.

The indicator used to detect acid formation was Bromothymol Blue, and the formation of gas was checked by floats placed in the medium. The pH of the medium was 7.2. Not a single culture formed gas during carbohydrate fermentation. Table 22 indicates the formation of gas on various sources of carbon. All experiments were repeated twice.

It will be seen that the various cultures of B.mycoides isolated from their natural habitat (many of which, with full justification, may be considered races) were very similar in biochemical properties. The overwhelming majority fermented glucose, levulose, maltose, and saccharose. The strictly oriented rugose variant, more often that the other variants, showed weak fermentation of saccharose, or complete inability to assimilate it.

TABLE 22

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RELATION OF B.MYCOIDES VARIANTS TO VARIOUS CARBON SOURCES

Culture	Number of Cultures	Glucose	Levulose	Galactose	Saccharose	Maltose	Lactose	Raffinose	Arabinose	Mannite
Ordinary, rugose										
From the soils of:										
Igarka	8	+	—	—	+[1]	+	—	—	—	—
Moscow	15	+	—	—	+[1]	+	—	—	—	—
Samarkand	1	+	—	—	+[1]	+	—	—	—	—
Frunze	1	+	—	—	+	+	—	—	—	—
Golodno Step'	1	+	+	—	+	+	—	—	—	—
Rugose, with annular structure										
From the soils of:										
Igarka	2	+	+	—	+	+	—	—	—	—
Moscow	2	+	+	—	+	+	—	—	—	—
Rugose, with strictly oriented strands										
From the soils of:										
Sumy	3	+	+	—	—	+ or —	—	—	—	—
Rostov on Don	3	—	+	—	—	—	—	—	—	—
	10	—	+	—	+[1]	+	—	—	—	—
Samarkand	3	+	+	—	+[1]	+	—	—	—	—
Frunze	1	+	+	—	+	+	—	—	—	—
Golodno Step'	1	+	+	+	+[1]	+	—	+	+	—
Intermediate forms										
From the soils of:										
Moscow	2	+	+	—[2]	+[1]	+	—	—	—	—
Frunze	11	+	+	—[2]	+[1]	+	—	—	—	—
Samarkand	2	+	+	—	—	+	—	—	—	—
	9	+	+	—[1] or +	+	+	—	—[1] or +	—[1] or +	—
Golodno Step'	2	+	+	—	+	+	—	—	—	—
Smooth type										
From soils of:										
Frunze	1	+	+	—	+	+	—	—	—	—
Golodno Step'	5	+	+	—	+	+	—	—	—	—

[1] Fermentation was slow with some of these cultures.
[2] One culture fermented galactose.
[3] Two cultures fermented galactose, raffinose, and arabinose.

In rare cases, cultures of B.mycoides were able to assimilate raffinose, galactose, and arabinose. Not one of the investigated cultures was able to ferment lactose and mannite.

Our collection of B.mycoides did not give indole nor hydrogen sulfide. Thus, all variants (rugose, transitional, and smooth) showed almost identical behavior toward carbon sources. It was only the strictly oriented rugose variants that did not ferment saccharose at all or weakly.

This work permits the conclusion that the fermentative properties of the natural variants of B.mycoides are affected less by environmental factors than the other properties of the bacterial cell.

Some investigators consider B.mycoides to be one of the strongest soil ammonifiers. This view is obviously erroneous. It is true that B.mycoides vigorously decomposes proteins, but Löhnis was able to demonstrate long ago that many asporogenic soil bacteria would then have to occupy the front row, if this criterion were decisive.

Individual authors give widely differing data as to the amounts of organic nitrogen converted by B.mycoides into ammonia. For example, according to /149
data by Holzmüller, only 4% of protein is converted into ammonia. Marchal (1894) stated that up to 46% of protein nitrogen was converted into an inorganic compound (NH_3). In media poorer in protein, this figure was appreciably higher.

Glinka-Chernorutskaya (1929) studied the ammonifying capability of B.mycoides in considerable detail. In investigations on the decomposition of peptone in the presence or absence of carbohydrates, she found that sugar retards the formation of ammonia. This was also confirmed by other investigators. Since many authors assumed that carbohydrates suppress the process of ammonification as a result of acid formation, Glinka-Chernorutskaya decided to check this proposition and found that an addition of chalk to the sugar medium did not increase the ammonia yield. This permits the conclusion that sugar is used preferentially by B.mycoides as a source of energy, and that therefore the decomposition of peptone is decreased. Waksman expressed a similar view, but Arnbeck objected to it.

On a casein-peptone medium, according to Glinka-Chernorutskaya, B.mycoides converts about 25% of the organic nitrogen into organic ammonia. Only in a single experiment was 32.1% of the nitrogen ammonified in nine weeks. She /150
also showed that filtrates of old cultures can be used rather successfully for growing B.mycoides. Media used seven times in succession still did not contain enough toxic matter to suppress the growth of B.mycoides. She also found that, on media obtained by the action of pancreatin on meat and containing diamino acids, B.mycoides gave good growth.

Media with monoamino acids did not give a satisfactory growth of B.mycoides.

Various authors reported that B.mycoides cannot utilize ammonium salts as a source of nitrogen. Grundmann, however, did grow B.mycoides on an ammonia-glycerol medium, where NH_3 was the only source of nitrogen. According to our own observations, far from all cultures of B.mycoides are able to grow on inorganic nitrogen. However, no laws governing the relation of individual vari-

ants of B.mycoides to nitrogenous nutrients have been discovered. In general, this species is not particularly exacting and develops on media containing extremely low concentrations of nutrients.

The proteolytic ferments of B.mycoides consist largely of endoenzymes. Their action is similar to that of the tryptases.

Another property of B.mycoides is its ability to reduce nitrates to nitrites.

According to Löhnis, B.mycoides has a slight ability to ferment urea and to decompose calcium cyanamide, under liberation of ammonia.

Sulfur can be oxidized in small amounts by B.mycoides (Demolon).

13. The Phenomenon of Bacteriophagy

The phenomenon of bacteriophagy in B.mycoides was first described by Cowles (1913) who detected the lysis of a culture under the action of a factor isolated from stagnant water.

The specific phage for B.mycoides was isolated by den Dooren de Jong in 1934. This phage was unable to induce lysis in a culture of bacteria related to B.mycoides.

Gordon (1940) showed that a phage antagonistic to B.mycoides also induces lysis of its variants resembling B.cereus.

Gordon considers this form, like B.fluorescens, B.albolactis, and B.Praussnitzii, to be a variety of B.mycoides. The phenomenon of bacteriophagy may also be utilized in defining the family relationships between B.mycoides and other bacteria.

Summarizing the material discussed in this Chapter, the following conclusions can be drawn: /151

1. The different variants of B.mycoides exhibit small, though analytically detectable, differences in cell width. The variants more often found in the North have a somewhat thinner cell than those inhabiting the South.

2. No comparative studies on the cell structure of the different variants has ever been made.

3. To identify a culture as B.mycoides, liquid media containing an 0.5 molar solution of magnesium salts can be used.

In the presence of magnesium, cultures of B.mycoides easily form vesiculose

cells (blisters). Other spore-bearing bacteria may give similar formations, but only in more concentrated solutions of magnesium salts.

4. The spores in the smooth forms of B.mycoides are somewhat larger than in the rugose forms.

There is some reason to believe that, in the different variants of B.mycoides, the germination of the spores does not proceed in an identical manner. However, no experimental verification exists.

5. Spores of southern cultures of B.mycoides, grown at an elevated temperature, are more resistant to heat. At the optimum temperature, the life cycle in the southern cultures is more rapid than in the northern cultures.

6. Of all bacteriological media, MPB may be used for identification of the variants of B.mycoides. On this medium, the rugose and smooth forms develop in an entirely different manner.

7. All variants of B.mycoides, isolated from various soils are completely identical with respect to their behavior toward carbohydrates. Only in the strictly oriented rugose forms does weak fermentation of saccharose occur more often than in the other forms.

No differences have been found, either, in the behavior toward sources of nitrogenous nutrients. B.mycoides preferably utilizes the nitrogen of organic compounds. Some cultures, however, are able to develop on ammonium salts.

8. The overwhelming majority of B.mycoides cultures yield sinistral colonies on MPA. The composition of their nutrient medium has a certain influence on the orientation of the bacterial filaments, but the variations discovered in this case are not of hereditary nature. Dextral cultures of B.mycoides were found only in mountain localities.

CHAPTER IV /152

DISTRIBUTION OF B.MYCOIDES IN THE SOILS OF VARIOUS CLIMATIC ZONES

1. Occurrence of Variants of B.Mycoides in Various Soils

After considering the features of the individual variants of B.mycoides found in nature, it is interesting to see how they are distributed in the soils of the various climatic zones. This will essentially answer the question as to the cosmopolitanism of these forms of bacteria, in the affirmative or negative.

The work of recent years on the ecology of B.mycoides has fully confirmed the conclusion of a number of authors (Grundmann, Kalinenko, and others) that this microorganism is far from being encountered in all soils. However, while the causes responsible for this phenomenon had not been discovered earlier, our own work disclosed a regular connection between the abundance of B.mycoides in a soil and the features of a certain type of soil-forming process. The scope of this Chapter does not permit a detailed discussion of this question, although it is of great theoretical interest. We are merely mentioning it here in our desire to note some of the difficulties encountered by investigators in the isolation of pure cultures of B.mycoides from a number of soil varieties. We have been able to isolate a rather large number of cultures of B.mycoides from widely differing soils of the Soviet Union, reaching from the Far North to the subtropics. Most of the soil specimens were taken by us personally.

The B.mycoides specimens were typed on peptone medium, and all experiments were run in triplicate. From the collection, we made pour cultures of well-studied typical growths, since certain variations in the composition of the various media might have a certain effect on the character of development of the strands in the colony.

The results are given in Table 23, which also indicates the classification of the isolated cultures with the described respective groups. Table 24 gives a summary of the occurrence of various types in individual soils. /154

This material shows that, in the northern zone, the most common forms are the ordinary rugose variants of B.mycoides. The forms giving colonies with elliptically curved strands on a peptone agar medium predominate in the northern chernozem and the mountain chernozem-like soils.

In the southern soils, there is a large population of the form with strictly oriented strands, which has never been encountered in the North. The number of ordinary rugose variants is relatively small here. A similar picture is /155 observed in the burozems of the South.

In the soils of the arid subtropics (serozems) we find a considerable variation in the subspecies of B.mycoides. Here, together with type 1c which is common to southern soils, cultures with elliptical coils are often encountered.

TABLE 23

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DISTRIBUTION OF B.MYCOIDES VARIANTS IN VARIOUS SOILS

Soil Type	Site of Sampling	Number of Cultures	Differentiation of Isolated Cultures, by Type						
			1a	1b	1c	2a	2b	3a	3b
Boreal zone									
Tundra soil	Near Igarka	12	10	2	—	—	—	—	—
	» Syktyvkar	15	15	—	—	—	—	—	—
	» Solikamsk	8	6	2	—	—	—	—	—
	» Moscow	23	18	3	—	—	2	—	—
		58	49	7	—	—	2	—	—
North Steppe zone									
Gray forest soils and degraded chernozems	Near Ryazan'	45	17	5	1	—	22	—	—
	» Uzonovo Stat.	9	3	2	1	—	3	—	—
	» Serebryanyy Prudy	38	7	2	6	13	9	1	—
	» Ranenburg	7	—	1	—	—	6	—	—
Northern chernozem	» Kursk	25	3	—	—	2	20	—	—
		124	30	10	8	15	60	1	—
Steppe zone									
Chernozem	Around Kharkov	16	—	—	16	—	—	—	—
	» Sumy	4	—	—	3	—	1	—	—
	» Shakhty	18	—	—	18	—	—	—	—
	» Millerovo	4	—	—	4	—	—	—	—
	» Rostov-on-Don	11	—	1	9	—	1	—	—
	» Nevinnomys	3	1	—	2	—	—	—	—
	» Armavir	6	—	—	6	—	—	—	—
	» Mineralnyy Vody	2	—	—	2	—	—	—	—
	» Krasnodar	12	1	—	11	—	—	—	—
	» Tbilisi	14	1	—	10	—	2	1	—
	» Yerevan	37	2	1	29	2	4	—	—
		127	5	2	109	2	8	1	—
Desert zone									
Gray earth (Serozem)	Golodno Step'	11	1	—	3	2	—	2	3
	Samarkand	20	1	—	4	6	9	—	—
	Frunze	6	—	—	2	3	—	1	—
		37	2	—	9	11	9	3	3

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Soil Type	Site of Sampling	Number of Cultures	Differentiation of Isolated Cultures, by Type						
			1a	1b	1c	2a	2b	3a	3b
Mountain soils									
Mountain tundra	Urals, polar .	5	5	—	—	—	—	—	—
Tundra-Alpine zone	» central . .	4	4	—	—	—	—	—	—
Alpine zone	» southern . . .	8	8	—	—	—	—	—	—
Mountain forest	» » . . .	2	1	—	—	1	—	—	—
Gray forest soil	» » . . .	14	1	—	—	7	5	1	—
Subalpine zone	Armenia, Alagez Mts.	2	2	—	—	—	—	—	—
Mountain-chernozem meadow soil	Near Sevan, (Armenia).	12	—	3	—	1	8	—	—
	Armenia, other sites	8	4	1	—	—	3	—	—
Subalpine meadow	South Kirgizia, near Dzhelal-Abad	4	3	—	—	—	1	—	—
Forest zone, nut trees	Same	17	3	—	—	8	5	1	—
Same, cultiv. field	» »	15	3	—	1	8	3	—	—
Same, virgin	» »	19	4	—	1	5	9	—	—
		110	38	4	2	30	34	2	—

It seems that these forms differ in several traits from the corresponding more northern variants of B.mycoides listed by us, but we still lack sufficient data to classify them as a separate type.

It is of interest that the soils of the southern mountain chernozems contain variants of B.mycoides identical with those met in the northern chernozems. In Armenia, already at altitudes of 1500 - 2000 m in the mountains, there is /156 a radical change in the forms of B.mycoides.

Thus, on the basis of the material already available, it can be stated that certain variants of B.mycoides belong essentially to certain soil zones. Detailed work on this question, of course, will require adequate preliminary study. It is probable that the extent of cultivation of the soils within the same zone will affect the existence of certain variants of B.mycoides in the soil. For example, in the zone of podzol soils, B.mycoides forms with elliptically curved strands were encountered only in the highly cultivated soils of the Timiryazev Agricultural Academy. Although there may be some complexity of the law formulated from soil background studies for the same type of B.mycoides, the basic propositions from the available material are so realistic that they must be considered reliable. The various data speak clearly against the hypothesis of vulgar cosmopolitanism among bacteria. On transition from one climatic zone to another, we encounter the replacement of one ecological type by another. Thus, each soil-climatic zone has its own dominant variants of B.mycoides.

TABLE 24

PERCENTAGE OF B.MYCOIDES VARIANTS IN VARIOUS SOILS

Zone	Proportion of Variants Encountered (in %)						
	1a	1b	1c	2a	2b	3a	3b
Boreal zone							
Tundra soils	92.6	7.4	—	—	—	—	—
Podzolic soils of the middle belt . . .	77.4	16.1	—	—	6.5	—	—
Northern Steppe zone							
Gray forest soils, degraded and northern chernozems	24.2	8.1	6.4	12.1	48.4	0.8	—
Steppe zone							
Chernozems	2.6	1.3	94.5	—	2.6	—	—
Burozems	5.9	2.0	74.4	3.9	11.8	2.0	—
Desert zone							
Serozems	5.4	—	24.2	29.8	24.3	8.1	8.1
Mountain soils							
Mountain tundra, Alpine and mountain-forest zones . . .	92	—	—	4	4	—	—
Gray forest soils, chernozem-like and burozem mountain soils	17.5	4.7	2.3	35.8	38.4	2.3	—

2. Distribution of B.Mycoides in Nature and in the Soils of the Soviet Union

B.mycoides is widely distributed in nature. This microbe usually inhabits the soil but is also found in ooze, in fluids of the intestinal tract of various animals, etc.

The first investigator of B.mycoides, Flügge, encountered this organism in almost all soils. Zimmermann (1891) and Marshall Word isolated B.mycoides from water. Emmerling (1897) attributed a certain role to B.mycoides in the phenomena of self-heating. Stocklaze (1898-99) found B.mycoides in the seeds of sugar beet which, after germination, developed sprout disease. König (1900) attributed a certain role to B.mycoides in the fermentation of tobacco, which, as is well known, was not subsequently confirmed.

According to Neide (1904), B.mycoides is encountered in horse dung, manure, and street dirt.

Harrison (1907) isolated B.mycoides from diseased potatoes.

Holzmüller isolated B.mycoides Flügge and B.mycoides δ from soil (from almost all soil specimens).

The remaining three strains of this bacterium described by him were found in the animal intestine. After repeated experiments, he also found B.mycoides β in the appendix of the guinea pig and B.mycoides γ in the stomach of the meal worm (the larvae of Tenebrio molitor). B.mycoides α was found in the stomach of the guinea pig and the gold fish, and B.mycoides Flügge in the intestine of the worm. /157

B.mycoides was not found in the small intestine of the rabbit, nor in the stomach of the larvae of flies nor of the Mermis worm.

Holzmüller postulated, on the basis of his observations, that certain forms of B.mycoides had adapted to life in a certain medium.

In general, it must be considered entirely correct to state, as Lehmann and Neumann have done, that B.mycoides is a soil bacterium. From the soil, it enters sources of water, food, and then passes into the contents of the intestinal tract.

Most of the above authors isolated B.mycoides from the soils, for their work.

B.mycoides is by no means present in all soils. For example, Kalinenko and Tauson were unable to detect B.mycoides in various mountain soils of the Pamir.

In an interesting study, Grundmann (1934) attempted to establish the laws of distribution of B.mycoides in soils. He studied about 260 soil samples of vertical zones of the high-mountain region and found that B.mycoides is present in larger numbers in fertilized cultivated soils than in virgin soils. In his opinion, the grazing of cattle, which contaminate the soil by their excrement, is reflected in the increase of B.mycoides in the soils. He established a relationship between the extreme altitudes of occurrence of B.mycoides and the grazing limits. His data give the impression that B.mycoides is encountered in high-mountain soils at only locations where there are domestic or wild animals.

At lower altitudes, the occurrence of B.mycoides is determined by many factors, among which the pH of the medium is in his opinion of considerable importance. Thus, in virgin soils on limestones, B.mycoides was found to altitudes up to 2400 m; on acid rock, this microbe was not encountered above 1800 m; the soils of the southern slopes of mountains contain more B.mycoides than those of the northern slopes.

The total number of cells of B.mycoides reached the maximum in the period of maximum development of the plant cover and declined toward the autumn. During the vegetation period, the ratio of spores to vegetative cells fluctuated considerably with climatic conditions. /158

Grundmann, together with the ordinary forms of B.mycoides, found smooth variants as well as a number of intermediate forms in the soils. He was unable to establish any relationship between soil type and character of the B.mycoides variants.

B.mycoides was found at higher altitudes on the southern slopes of the mountains than on the northern slopes.

Our own experience has shown (see below) that the distribution of B.mycoides has to do with the type of soil-farming process rather than with the presence of animals on a given territory. In particular, meadow soils have a rather abundant flora of B.mycoides, but since animals are usually confined to the meadow zones, Grundmann might have been induced to believe that B.mycoides was an imported organism. Actually, the situation is different, which will be clear from his data. For example, Grundmann noted that, on the high northern slopes where B.mycoides is rarely found, an accumulation of organic matter is responsible for multiplication of this microbe.

Certain data on the distribution of B.mycoides in various soils were given by Novogrudskiy and Kononenko. They make it obvious that B.mycoides is easier to isolate from some soils, such as the podzols, than from others (Table 25). The cause of this phenomenon was not given by these authors.

TABLE 25

OCCURRENCE OF B.MYCOIDES IN VARIOUS SOILS
(According to Novogrudskiy and Kononenko)

Soil Type	Total Number of Soils Studied	Soils in which B.Mycoides was Found		Cultures of Various Types Isolated from these Soils		
		Present	Absent	Medium	Thick	Thin
Podzols	6	6	—	8	3	1
Chernozems	6	4	2	3	—	2
Chestnut soils	2	—	2	—	—	—
Solonets	1	—	1	—	—	—
Solodi	1	—	1	—	—	—
Krasnozems	1	1	—	1	5	—
Serozems	1	—	1	—	—	—

No broad generalizations can be drawn from the work by Novogrudskiy and /159
Kononenko, since they only studied relatively few soils.

Our own studies, whose results we will discuss in the next Chapter, permit a definite conclusion as to the abundance of B.mycoides in various soils and as to the causes responsible for the enrichment of soils in this microorganism.

We have reached the conclusion that B.mycoides multiplies in a given soil only during certain periods of decay of organic matter. In various soils the mineralization of organic residues proceeds at various rates, which explains the enrichment or pauperization of a soil in B.mycoides. Since, on the whole, the climatic conditions determine the trend of the soil processes, and thus also the formation of the soil type, it is only natural that different types of soil should show a different abundance of B.mycoides.

Table 26 contains selected data that distinctly characterize the various soil types, with respect to the abundance of B.mycoides. This material shows the percentage of soil clods in which B.mycoides germinated on a solid nutrient medium. In most of the analyses, we used MPA and potato agar. On the latter medium we often observed a better growth of B.mycoides in soil clods, especially in cases where the soil contained smooth variants with depressive growth. On potato agar, B.mycoides gave a more diffuse growth and, especially in the case of smooth variants, facilitated the elimination, from the zone of antagonistic influence, of other bacteria that developed around the soil clod (Fig.76).

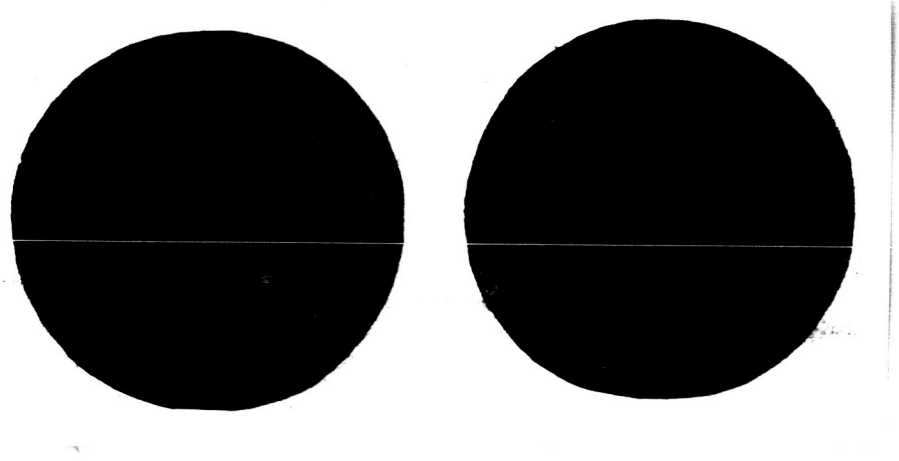

Fig.76 Isolation of B.Mycoides from a Serozem Soil
by the Crumb Method
Left - Cultivation of soil crumbs on MPA; Right - Cultivation
of crumbs on potato agar

It is clear from these data that the virgin soils of the tundra, except for those on a floodplain, are extremely poor in B.mycoides. An inoculation of the culture with fresh soil samples from forest land gradually and slowly increased the abundance of B.mycoides.

This picture apparently is easily explained by the consumption of organic matter, taking place under northern conditions. The low mineralization of plant residues constitutes a poor environment for the growth of B.mycoides.

The cultivation of soils, which artificially improves the microbiological processes, causes an increase in the number of B.mycoides in the soil.

TABLE 26

GERMINATION OF B.MYCOIDES IN SOIL CRUMBS

Soil Type	Soils Investigated	Percent of Soil Crumbs with B. Mycoides Colonies	
		On MPA	On Potato Agar
1	2	3	4
Tundra soils (near Igarka)	Forest subsoil: Sample № 1	0	0
	Sample № 2	0	0
	Floodplain soil	86	100
	Soil of newly cultivated field after clearing the forest, planted to cereals	5	3
	Same, planted to oats	0	0
	Same, planted to potatoes	0	0
	Soil cultivated for 7 years before time of analysis, planted to cereals	24	20
Soils of podzolic zone	Cultivated soils of Timiryazev Agricultural Academy	82—92	86—95
	Virgin soil of floodplain meadow near Kolomna	94	100
	Oats crop, near Kolomna	100	100
	Sowing of clover, 1st year, same place	100	100
	Sowing of clover, 2nd year, same place	100	100
	Sowing of clover, 3rd year, same place	93	100
	Fir forest, at Korenev (near Moscow)	97	100
	Mixed forest, same place	95	100
	Cleared forest, same place	3	5
	Sowing of potatoes, same place	100	100
	Sowing of serradella, same place	60	85
Soils of chernozem zone	Cultivated soil, Sumy Experimental Station	43—59	29—44
	Field of alfalfa, Kutuluk, Kuybyshev Oblast'	0	—
	Wheat field, same place	0—25	—
	Oats field, same place	0	—
	Barley field, same place	0—12	—
	Forest soil, same place	65	—
	Virgin meadow soil, same place	0—5	—
	Virgin soil, solonetz	0—21	—
Chestnut soils	Personal subsidiary plot at Arosan (Kirgizia) virgin soil	0	7
	Personal subsidiary plot, virgin soil in brook	16	80
	Same, irrigated wheat	12	80
	Same, irrigated potatoes	0	35
	Same, wheat, barrel-watered	0	13
	Yur'yevka Collective Farm, floodplain wheat	0	18
	Same, corn	0	12
	Same, virgin	0	0
	Pishpek (Kirgizia) Seed Selection Station, virgin	0	0
	Same, perennial grasses	15	35
	Same, manured garden soil	55	80
	Experimental Station (Kirgizia) virgin soil	0	0
	Same, sowing of watered corn	15	35
	Same, sowing of watered alfalfa	81	90
Serozem soils	Golodno Step'. Pakhta-Aral, sowing of watered alfalfa	0	0
	Same, sowing of watered rye grass	0	0
	Same, sowing of watered cotton, 1st year	5—22	10—53
	Same, sowing of watered cotton, 2 - 3 years	0	0

Floodplain soils are always distinguished by more favorable conditions, which is the explanation for the greater abundance of B.mycoides.

Soils of the podzol zone, both cultivated and virgin, contain a considerable number of B.mycoides. It is interesting to note that even forest soils /160 of rather acid reaction likewise contain B.mycoides, which evidently grow on the particles of decayed plant tissues with a more neutral reaction.

The climatic conditions of the podzol zone result in a rather moderate rate of microbiological processes. Mineralization of the organic residues and of the products of bacterial synthesis proceed at moderate speed, which results in the persistence of favorable growth environments for B.mycoides, over prolonged periods of time.

In the zone of chernozem and chestnut soils, there again is a depletion of B.mycoides. Our data indicate that the Ukrainian chernozems, in the vicinity of the City of Sumy, are richer in B.mycoides than the chernozems of the eastern regions (Kutuluk). The causes retarding the growth of B.mycoides differ from those in the Far North, since the conditions in the chernozem zone are favorable to growth, specifically from the thermal aspect. Apparently, the mobilization of the highly mobile organic residues in the chernozems is more rapid, thus shortening the period of favorable conditions for the existence of B.mycoides in the soil. Of course, during the various seasons, the soil may contain more or less numbers of B.mycoides; however, in general the abundance will be less than in the podzolic zone. Here, the features of irrigation and water supply, the type of relief, the stands, etc. may also affect the result. Consequently, it is no surprise that in some cases (for instance, in the forest soils of the /162 chernozem zone) B.mycoides is relatively abundant.

Because of this, the signs of occurrence of B.mycoides in southern soils are higher on potato agar than on MPA.

In serozem soils, B.mycoides is found still less frequently, mainly /163 after the plowing-under of grasses, when the soil is enriched by a large amount of organic matter. Apparently, the temperature conditions of serozem soils further shorten the period of decomposition of plant residues. Thus, the period of intense growth of B.mycoides in the soil is also substantially shortened. This means that the stress of the mineralization process determines the extent of occurrence of bacteria of the B.mycoides group in the soil.

The influence of climatic conditions on the composition of bacterial associations in the soil will be considered in greater detail in the last Section of this book.

CHAPTER V /164

GROWTH TEMPERATURE OF THE GEOGRAPHICAL RACES OF B.MYCOIDES

1. Location of Optimum and Maximum Temperature for the Geographical Races of B.Mycoides

One of the problems of this study was to establish the existence of an adaptive reaction in soil microorganism to the temperature condition of the climate. This phenomenon is easiest to illustrate on the example of a microorganism considered a cosmopolite, whose identification involves no controversial points.

Such forms of microorganisms, in particular among bacteria, are known to be relatively few. Of these we selected B.mycoides, for the reasons noted in the introductory Chapter.

We decided to verify the growth rate curves of individual cultures of this microorganism isolated from various localities of the USSR. If temperature shifts were discovered, we could then attempt to establish a correlation between the indices of the climate and the main temperature points characterizing the growth of such bacteria. This would make it possible to confirm the general occurrence of adaptive reactions, by studying the growth temperatures of other soil bacteria.

However, the variants of B.mycoides, each of which is encountered mainly in a certain climatic zone, were found to differ in their principal temperature points. A similar phenomenon was noted with respect to the osmotic properties of the cell. These traits are firmly fixed and transmitted to the progeny. Thus, the ecotypes of B.mycoides differ among themselves in a number of essential traits, permitting their classification as races. However, temperature adaptation is a distinct trait of all B.mycoides variants of any soil-climatic zone.

In studying the relation of B.mycoides with various temperatures, we /165 used the giant-colony method. We also used this method, which is particularly convenient in studying bacteria with a diffuse growth, on other microorganisms.

Our work was usually done in the following sequence: A pure stab culture was made on a cooled agar medium in a Petri dish. The material used for inoculation was a water emulsion of a culture prepared from a one-day agar culture. After a certain time, during which the infected Petri dishes were kept in the incubator at a certain temperature, the size of the colonies was measured with a ruler. Each experiment was run in triplicate.

Before selecting this method of rating the growth of the bacteria, we also tried other methods. For instance, we attempted to culture the bacteria in a liquid nutrient medium and to measure the number of cells by the method of transfer, or by counting in a Thoma-Zeiss chamber.

For cultures giving a uniform clouding of the medium, it was easy to estimate the effect of temperature on bacterial multiplication by this method. It is true, as shown by experiments, that the estimate by culturing did not yield substantially different data according to the measuring method. For a number of cultures, we obtained an exactly identical growth rate curve, no matter whether we used the culturing method or measured the size of the colonies formed at various temperatures.

For an analysis of cultures forming pellicles on MPB, the counting becomes extremely difficult. We attempted to break up the resulting pellicle, but even this did not help very much. The counting of the cells in the emulsion with a Zeiss chamber was likewise very difficult.

Since many soil bacteria, including B.mycoides, form pellicles when grown on liquid nutrient media, we decided to drop both the inoculation method and the use of the Zeiss chamber.

An attempt to use the method of centrifugation, employed by Kron in estimating the mass of multiplying cells, was also unsuccessful. The bacterial films were badly packed in the constricted part of the centrifugation tube, and the measurements could not be accepted as entirely reliable. The last of the methods mentioned can be used successfully only on cultures giving a uniform clouding in the liquid nutrient medium.

All these factors induced us to select the method of giant-colony measurements, although various objections could be raised against this. Since this /166 method is more reliable for bacteria with large colonies, we preferred to select cultures giving colonies with a diffuse growth. Of course, it goes without saying that B.mycoides is one of the first such microorganisms that would come to mind.

The giant colonies of B.mycoides on MPA were usually measured a day after inoculation of the medium, and the arithmetic mean of the measurements was taken. In the absence of growth, the observations were continued on the following days.

In this project, we studied several cultures of B.mycoides isolated from soils of the following localities of the USSR:

Region of Igarka (Siberia, northern podzols).
" " Arkhangel'sk (European part of USSR, northern podzols).
" " Omsk (Siberia).
" " Moscow (zone of podzol soils). .
" " Kharkov (zone of chernozem soils).
" " Nosovka (zone of chernozem soils).
" " Sumy (zone of chernozem soils).
" " Don region (zone of chernozem soils).
" " Krasnodar (zone of chernozem soils).
" " Central Asia (zone of serozem soils).

Thus, our collection of various cultures of B.mycoides was taken from highly contrasting soils formed under completely different climatic conditions. We had available cultures of B.mycoides from both the most northerly localities

TABLE 27

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DEVELOPMENT OF INDIVIDUAL CULTURES OF B.MYCOIDES AT VARIOUS TEMPERATURES

Site of Culture Isolation	Relative Size of Colonies at Temperature (in °C)											
	18	26	28	30	32	35	36	37	40	42	44	46
Vicinity of Arkhangel'sk												
Culture 1	6.7	100		94	53	4.1		0.0				
2	9.4	100		88	73	10.2		0.0				
3	11.9	76.2		90	52.4	3.4		0.0				
4	9.0	84.0		100	58.0	1.0		0.0				
5	8.8	100		93.3	82.2	1.1		0.0				
6	5.9	100		76.6	54.2	2.3		0.2				
Average	8.6	92.3		89.9	63.8	3.6		0.03				
Vicinity of Igarka												
Culture 1	17.1	100	77.1	80.1		10.0		0.0				
2	10.5	100	83.3	77.7		16.6		0.0				
3	12.3	100	79.2	66.1		25.7		0.0				
4	14.2	100	82.9	42.6		21.7		0.0				
5	7.6	80	100	92.0		15.6		0.0				
6	13.1	100	92.5	72.9		26.3		0.0				
Average	14.1	96.6	85.8	71.9		17.6		0.0				
Vicinity of Moscow												
Culture 1	47.2	100		91.4	72.8		0.6	0.0	0.0			
2	24.8	75.5		100	63.8		1.4	0.0	0.0			
3	27.2	72.1		100	81.2		1.1	0.0	0.0			
4	26.0	86.6		100	82.9		0.6	0.0	0.0			
5	18.3	75.6		100	60.4		27.3	20.8	0.0			
6	19.8	54.3		100	72.8		50.1	43.4	32.6	0.0		
7	7.6	65.2		100	73.3		3.8	0.0	0.0	0.0		
8	10.2	100		88.9	66.6		31.2	22.2	9.9	0.0		
9	10.2	26.5		100	52.9		41.3	26.5	17.7	0.0		
10	8.3	68.7		100	68.7		3.2	0	0	0		
11	11.4	38.1		100	61.9		2.3	0	0	0		
12	7.8	46.1		100	96.1		7.6	0	0	0		
13	5.6	35.5		100	91.4		3.1	0	0	0		
14	11.2	29.4		100	76.4		1.4	0	0	0		
Average	17.1	62.4		98.6	73.4		12.5	8.1	4.3	0.0		
Vicinity of Kharkov												
Culture 1	8.7	79.3		84.6	100	94.6		70.3	3.3	0.0		
2	10.2	45.5		73.1	100	76.4		66.0	2.5	0.0		
3	6.6	77.6		95.3	100	83.2		75.7	1.9	0.0		
4	2.5	49.2		100.0	68.3	79.2		55.8	3.0	0.0		
5	9.2	52.5		88.3	100	72.5		59.2	3.2	0.0		
6	9.7	64.9		75.0	100	72.6		66.1	3.6	0.0		
Average	6.8	62.0		86.1	97.4	79.7		64.6	3.3	0.0		

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Notes: 1. The figures given in the Table are the averages of 2 - 3 measurements. 2. The colonies were usually measured a day after inoculation.

TABLE 27 (cont'd)

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Site of Culture Isolation	Relative Size of Colonies at Temperature (°C)											
	18	26	28	30	32	35	36	37	40	42	44	46
Nosovka Experimental Station												
Culture 1	6.4	61.4		100	82.8	77.1		52.8	11.1	0.0	0.0	
2	10.1	53.1		95.4	100	84.3		43.1	6.1	0.0	0.0	
3	7.8	50.0		96.0	100	75.8		57.0	8.0	0.0	0.0	
4	8.6	48.9		44.9	100	92.4		96.5	24.1	6.9	0.0	
Average	8.2	58.3		84.1	95.7	94.1		60.6	12.5	1.7	0.0	
Sumy Experimental Station												
Culture 1	10.2	44.3		63.9	100	79.8		38.1	0.0	0.0	0.0	
2	8.4	70.0		73.3	100	89.6		95.3	35.5	6.0	0.0	
3	6.3	91.9		85.7	100	96.3		96.6	61.3	8.0	0.0	
4	6.8	19.1		100	77.9	69.4		58.6	26.0	4.0	0.0	
5	9.6	22.0		70	100	85.4		66.0	8.0	0.0	0.0	
Average	8.3	49.5		78.6	95.6	84.1		69.9	24.7	3.6	0.0	
Don District												
Culture 1	1.1	93.3			97.7	83.3		24.4	0.4	0.0	0.0	
2	2.0	85.4			74.5	100		94.5	29.1	4.0	2.7	
3	2.1	59.7			70.8	100		66.6	11.1	2.1	0.3	
4	19.5	85.1			100	50.0		32.6	0.0	0.0	0.0	
5	5.5	89.3			83.3	100		48.1	0.9	0.4	0.0	
Average	6.0	82.6			85.3	88.7		53.2	8.3	1.3	0.6	
Krasnodar District												
Culture 1	1.4	67.3			—	100		—	85.7	21.4	0.0	
2	0.8	72.7			74.2	72.7		100	22.7	2.5	0.0	
3	0.4	45.3			93.5	100		91.3	26.1	11.9	0.4	
4	0.0	17.0			48.9	100		76.6	15.9	4.3	0.4	
5	0.0	77.0			31.9	90.1		100	85.2	23.4	3.3	
6	0.0	60.6			80.3	86.9		100	26.2	3.7	0.0	
7	0.0	76.2			79.6	100		98.1	20.5	0.0	0.0	
Average	0.3	59.4			66.9	92.8		80.8	40.3	11.3	0.6	
Central Asia												
Culture 1 from near Tashkent		54.0		80.0	82.1			100	33.0	9.4		0.0
2 » » »		24.2		36.4	81.8			100	60.6	31.4		0.0
3 » » »		57.4		74.5	74.5			100	46.8	17.2		0.0
4 » » »		52.1		66.6	73.2			100	46.6	20.0		0.0
5 » » »		34.1		41.7	62.5			100	95.8	45.7		0.0
6 » » »		22.5		78.3	78.3			100	23.5	35.4		0.0
Average		40.7		61.1	75.4			100	61.9	27.5		0.0

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TABLE 27 (cont'd) /171

Site of Culture Isolation	Relative Size of Colonies at Temperature (°C)											
	18	26	28	30	32	35	36	37	40	42	41	46
7 Golodno Step' . .		24.7	49.8		79.3		99.9	100	67.9	0.0		0.0
8 » » . .		13.2	41.7		81.2		100	53.9	33.4	0.8		0.0
9 » » . .		17.5	48.7		71.3		97.5	100	47.6	15		0.0
10 » » . .		7.0	58.1		69.4		80.6	100	90.3	51.5		0.0
11 » » . .		21.0	51.1		74.3		93.8	100	89.9	55.3		0.0
12 » » . .		16.6	52.1		66.7		76.0	100	88.8	32.0		0.0
Average	sl.	28.9	50.2	62.9	74.5		90.6	96.1	65.7	26.1		0.0
Western Siberia												
Culture 1	18.3	86.7			100	2.9		0.0				
2	16.0	70.2			100	1.6		0.0				
3	14.0	71.4			100	1.7		0.0				
4	19.2	100			64.3	2.4		0.0				
Average	16.9	82.1			91.1	2.1		0.0				

of the USSR and from burning deserts of Central Asia. We were certain that, on this material, we would be able to detect the existence of an adaptive reaction if and when it occurred.

As obvious from Table 27 (containing selected data), the experimental work led to interesting results. On comparing the tabulated values for each culture, the size of the colony at the optimum growth temperature was taken as 100. We then determined, for each size, the percentage of this size and the absolute size of the colonies at various temperatures.

This approach was adopted because our work extended over several years. Therefore, the individual cultures of B.mycoides were not studied at the same time; thus, the resultant minor deviations in the composition of the media and the time of incubation naturally prevent a comparison of the absolute size of the colonies. Taking the adopted work-up as basis, the experimental data can be reduced to a common denominator.

We also did supplementary work on the question, of independent interest, /172 as to the rate of multiplication of the various temperature races of B.mycoides. This will also be discussed below.

Returning to the data of Table 27, it can be demonstrated that, as one progresses from North to South, the optimum and maximum temperatures of the individual cultures of B.mycoides show an appreciable rise. For example, the optimum temperature for cultures isolated from the northern podzols is near 26-28°C,

for those from soils of the main podzol belt, it is near 30° C, for those from soils of the chernozem belt it is near 32 - 35° C, and for those from soils of the serozem type, near 38° C.

It must be noted that even individual cultures of B.mycoides, isolated from the same locality exhibit individual peculiarities. For example, most of the Moscow cultures stop growing at 37 - 38° C. However, we also find forms of these cultures that grow still fairly well at this temperature.

In general, B.mycoides with a higher maximum temperature are encountered in highly cultivated soils. On this basis, we are inclined to consider them forms introduced into the soil together with the manure, which is rich in thermophilic and thermotolerant forms of microorganisms.

Individual deviations in various cultures of B.mycoides, with respect to the optimum temperatures, have also been observed. Nevertheless, the material of Table 27 shows the sharp difference of the growth rate curves for the various geographic races of B.mycoides.

This refers not only to the above optimum growth temperatures but also to the maximum temperatures. Most cultures of B.mycoides stopped growing at the following temperatures:

From northern podzols	at 36 - 37° C
From the podzol belt	at 37 - 40°
From the northern chernozem belt	at 42° C
From the southern chernozem and serozem belts	at 44 - 45° C

A certain adaptation to lower temperatures is also noted in the more northerly races of B.mycoides. Whereas, at 16° C, cultures isolated from the northern zone yield perceptible colonies in one day, the more southerly forms show either a weaker growth (in cultures from chernozems) or complete absence of growth (in cultures from serozems).

Thus, the above material as a whole permits the definite statement that /173 B.mycoides does have a certain adaptive reaction to climatic conditions.

Our considerable experience with B.mycoides permits speaking of the stability of this trait in the individual cultures of this microbe. Experimental cultures were grown by us for a long time under identical conditions, but this produced no change at all in the growth-temperature curves characteristic of the particular race. This means that the relation to temperature is a stable trait of the individual cultures of B.mycoides, and is transmitted to a long string of progeny. It can thus be stated that B.mycoides, evidently like most soil microorganisms, has geographical races that differ primarily in their growth temperatures. As demonstrated below, the geographical races of soil bacteria differ not only in growth temperature but also in specific osmotic pressure, in addition to a number of the cultural traits described above.

This proposition does not eliminate the question as to the possibility of artificial or natural mutation of microorganisms. We merely emphasize the con-

siderable stability of the properties inherent even to relatively simply organized lower organisms, such as bacteria.

In concluding this Section, we present a schematic diagram (Fig.77) which clearly shows the shift in the growth temperature for the individual geographical races of B.mycoides.

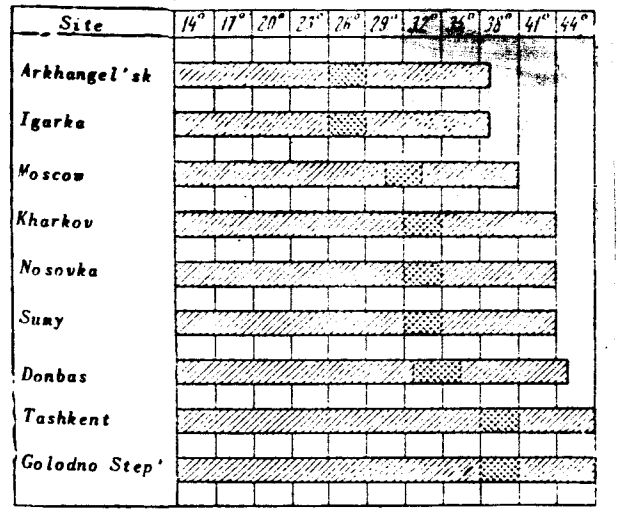

Fig.77 Position of Optimum and Maximum Temperature Points for Various Races of B.Mycoides (The double hatching indicates the position of the temperature optimum)

2. Energy of Multiplication and Biochemical Activity of Geographical Races of B.Mycoides

In our subsequent work on the geographical races of B.mycoides, we compared the development of several cultures under completely identical conditions with respect to temperature and incubation time.

For technical reasons, we were unable to make a simultaneous experiment on a large number of cultures; consequently, we limited our study to the most contrasting geographical races of B.mycoides. We selected for this study the Northern Igarka races, some cultures from the chernozem belt (Sumy) and cultures from the serozem soils of Central Asia (Golodno Step').

This resulted in an interesting fact. The southern races of B.mycoides not only showed a shift in optimum temperature toward higher values but also /174 multiplied far faster at the optimum temperatures than did the northern races. For example, the Igarka cultures formed colonies of 1.0 - 1.5 cm diameter after one day on MPA at their optimum temperature, while the Sumy cultures yielded colonies of 2.5 - 3.5 cm diameter and the Central Asian cultures, of 3.6 - 4.4 cm. Since the rate of development of a colony indicates the vigor of reproduction of

a bacterium, the important conclusion can be drawn that the southern geographical races multiply more rapidly at the optimum temperatures than the northern. The biochemical activity of microorganisms is usually associated with the energy of growth of the mass of bacterial cells. Consequently, there is every reason to believe that the southern races of bacteria have stepped-up life processes in a favorable environment. This apparently explains the surprising rate of decomposition of organic matter observed in the serozem soils of Central Asia as compared to its preservation in northern soils.

TABLE 28

FORMATION OF GIANT COLONIES BY GEOGRAPHICAL RACES OF B.MYCOIDES AT VARIOUS TEMPERATURES
(Size of One-Day Colonies on MPA, in cm)

Culture		Growth Temperature, °C				
		16	26	30	38	42
Igarka	1	0.6	1.2	1.0	0.0	—
	2	0.4	1.0	0.95	0.0	—
	3	0.6	1.5	1.1	0.0	—
	4	0,5	1.5	1.2	0.0	—
	Average	0.5	1.3	1.1	0.0	—
Sumy	1	0.4	1.8	2.5	0.45	0.0
	2	0.35	2.4	2.4	0.4	0.25
	3	0.6	1.8	3.5	2.1	0.30
	Average	0.4	2.0	3.1	1.3	0.2
Central Asian	1	0.1	1.0	2.9	4.4	1.5
	2	0.2	1.1	2.7	4.3	2.2
	3	0.05	0.8	2.1	3.7	1.4
	4	0.05	0.75	2.3	3.6	0.5
	5	0.1	0.8	2.3	3.7	1.2
	Average	0.1	0.9	2.5	3.9	1.4

It should be mentioned in this connection that various authors have noted a decreased activity of bacteria isolated in the North. Thus Isachenko, in /175 studying bacteria of the Arctic Ocean, found a very weak biochemical activity in nitrifying and nitrogen-fixing bacteria. On the other hand, Petrosyan isolated nitrogen-fixing bacteria from Armenian soils that fix atmospheric nitrogen at a vigorous rate.

Without denying that bacteria of very different activity can be isolated from any soil specimen, our own experiments have convinced us that the above general law does exist.

Table 28 compiles factual material from these experiments. Obviously, whereas the southern races develop with considerably more vigor at optimum temperature, they show a much slower growth at lower temperatures (16 - 26° C) than do the northern races.

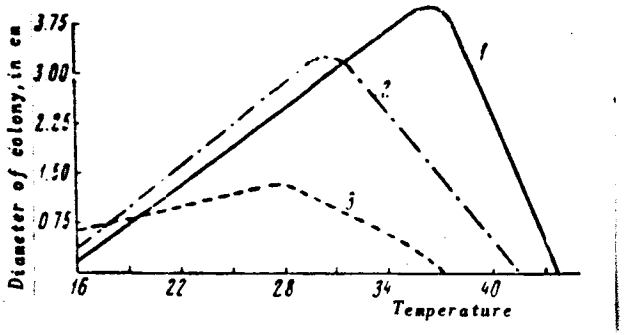

Fig.78 Variation of Colony Size among Geographical Races of B.Mycoides at Various Temperatures

We believe that the potential biochemical activity of the geographical races of B.mycoides, to a certain extent, can be characterized by the areas /176 under the curves plotted on the basis of the average values given in Table 28.

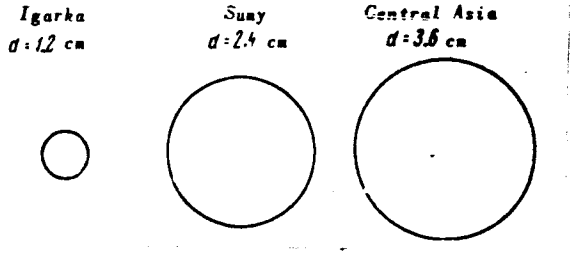

Fig.79 Size of One-Day Colonies of the Geographical Races of B.Mycoides

As indicated in Fig.78, the curves for the southern races delineate a considerably larger area than those for the northern. Taking the area under the curve for the Igarka culture as 1.0, the area for the Sumy cultures must be taken as 2.5 and that for the Central Asian cultures, as 3.0.

A series of experiments, analogous to those just discussed, confirmed the regularity of this phenomenon. In particular, we were able to demonstrate that the increase in the size of the giant colonies depends on the number of cells formed, and that this number usually increases more than the diameter of the colonies.

Figure 79 graphically compares the size of the giant colonies of various ecological races of B.mycoides at their optimum temperature.

Because of the greater growth of the cell mass at optimum temperature in southern races of B.mycoides, these subspecies are also able to do more biochemical work. Thus, on potato agar the Igarka cultures of B.mycoides give /177 a hydrolysis zone of 0.8 - 1.2 cm diameter within one day, while the Central Asian cultures form a zone of 2.4 - 3.5 cm diameter (Table 29).

TABLE 29

HYDROLYSIS OF STARCH AT OPTIMUM TEMPERATURES, BY VARIOUS GEOGRAPHICAL RACES OF B.MYCOIDES

Culture	Temperature, °C	
	26	37
1. Size of giant colonies (in cm)		
Igarka 3	1.6	0.0
133	1.8	0.0
79	1.6	0.0
Central Asian 32	1.3	4.1
29	1.2	3.5
2	1.7	3.7
2. Size of hydrolysis zone (in cm)		
Igarka 3	0.9	0.0
133	1.0	0.0
79	0.75	0.0
Central Asian 32	0.8	3.2
29	0.6	2.6
2	0.7	3.0

In exactly the same way, giant colonies of the southern races of B.mycoides, grown at optimum temperature and taken up together with the agar, give a considerably greater catalase activity than the giant colonies of northern races (Table 30). In the same determinations, the colonies cut out together with the agar were triturated in water in a porcelain mortar, and the catalytic activity of the water slurry was determined in a Liebig-type apparatus.

This phenomenon is only natural, since the catalase content of a culture, to a certain extent, is proportional to the number of cells, while the bacterial mass, as already noted, accumulates more actively in the southern cultures. It is obvious, however, that there may be considerable deviations of individual character in various cultures of B.mycoides; as a whole, this does not impair the general law established.

These data show that, with increasing optimum temperature of the geo- /178 graphical races of B.mycoides, their reproduction rate and the biochemical activity of their culture also increase. This fully confirms Imshenetskiy's view that organisms of thermophilic type have a high impetus of vital processes.

TABLE 30

CATALASE ACTIVITY OF BACTERIAL MASS OF GIANT COLONIES OF GEOGRAPHICAL RACES OF B.MYCOIDES

Culture		Size of Giant Colonies at Temperature, °C		Catalase Activity of Colony (O_2, in cm)	
		26	37	26	37
Igarka	3	0.8	0.0	0.25	0
	79	1.2	0.0	0.12	0
	133	1.2	0.0	0.12	0
Central Asian	2	1.4	3.5	0.07	1.8
	32	1.0	2.7	0.05	2.67
	29	1.0	2.4	0.15	3.24

Note: The quantity of oxygen formed is obtained after subtracting the volume of gas formed in the control with sterile agar.

In concluding this Section, it should be mentioned that the above material does not answer the question as to the activity of an individual cell of a given culture. It would be logical to assume that the cells of the northern races of B.mycoides, adapted to life in a severe environment, would have more active ferments than the southern races. As demonstrated by the data presented below, this hypothesis is fully confirmed by the study of the catalase of various B.mycoides cultures.

3. Enzymatic Activity of Cells of B.Mycoides Geographical Races

Several authors (Ivanov, Kursanov, Grebinskiy, Wager) reported that the oxidative system is less active in southern plants than in the same forms growing in the North. This variability is without doubt adaptive.

It must be assumed that a reorganization of the type mentioned by us refers not only to the oxidative system of the organism but also to other aspects of the enzymatic activity of the organism. For the time being, this hypothesis has been demonstrated on the typical example of catalase and peroxidase.

In connection with our work on the geographical variability of B.mycoides, we checked on variation of catalase activity in a number of ecological races /179 of this bacterium. As we will show, we found full confirmation of the law established earlier for higher plants. Individual cells of northern cultures of B.mycoides decomposed hydrogen peroxide considerably more vigorously than cells of southern cultures.

Passing now to a discussion of the factual material obtained in our work, we would first like to describe briefly the method for determining bacterial catalase.

Our work was done in a simple apparatus, specially designed for this purpose.

The cultures of B.mycoides were grown at 30° C on meat-peptone agar, containing 2% peptone. The high concentration of peptone strongly inhibited sporulation and made it possible to restrict the work to cultures containing only vegetative cells.

While most cultures of B.mycoides, on ordinary meat-peptone agar, produce spores at 30° C as early as on the second day, no sporulation was noted even after four days on increase in the amount of peptone.

The cultures grown on the surface of meat-peptone agar were removed by a spatula, thoroughly emulsified in physiological salt solution, and filtered through filter paper to remove remaining lumps. The concentration of the cells was determined with a counting chamber. Usually, our suspensions contained 120 - 150 million cells per cubic centimeter. The results were converted to terms of one billion cells.

The age of the culture was accurately checked, since the activity of the cells declined sharply with age.

For all these cultures, the catalase activity was determined at 20° C. This was done after we had found that the 5-min counts usually employed by us did not change greatly when varying the temperature in the range from 20 to 35° C. The influence of temperature on the index of catalase activity was verified for both the northern and southern race (Table 31). A rise in the temperature to 42° C already caused a marked inactivation of the catalase, especially in the northern races.

TABLE 31 /180

EFFECT OF TEMPERATURE ON CATALASE ACTIVITY INDEX

Temperature of Experiment (in °C)	Amount of Oxygen (in cm^3) Produced by One Billion Cells					
	Central Asian Culture			Igarka Culture		
	In 5 min	In 10 min	In 15 min	In 5 min	In 10 min	In 15 min
22	2.9	4.6	5.9	21.0	23.1	23.8
34	3.0	4.6	5.1	21.5	22.2	23.6
42	2.1	3.1	3.3	0.7	1.3	1.7

After these remarks of a methodological character, we give, in the summary Table 32, data characterizing the catalase activity of several cultures of B.mycoides isolated from soils of various climatic zones of the USSR.

TABLE 32

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CATALASE ACTIVITY OF VARIOUS CULTURES OF B.MYCOIDES

Zone of Isolation of Culture	Place of Isolation of Culture	Character of Isolated Culture	Quantity of Oxygen Produced by One Billion Cells		Average Values	
			In 5 min	In 10 min	In 5 min	In 10 min
Northern belt of the USSR	Near Igarka	Ordinary rugose culture				
		1	23.1	29.4		
		2	26.1	30.3		
		3	21.3	24.6		
		4	14.6	21.9		
		5	14.0	20.3		
		6	10.7	22.8		
		7	24.0	28.5		
		8	23.9	29.5	20.5	26.7
	Near Syktyvkar	1	18.6	26.2		
		2	21.6	31.0		
		3	23.6	32.6		
		4	23.8	26.4		
		5	24.8	29.1		
	Near Solikamsk	1	29.1	40.0		
		2	11.4	16.4		
		3	13.6	20.4		
		4	16.6	24.6		
Central belt of the USSR	Moscow suburbs	Ordinary rugose culture				
		1	9.4	21.0		
		2	19.2	30.8		
		3	4.7	7.8		
		4	19.6	26.0		
		5	9.8	13.8		
		6	15.4	27.6		
		7	9.4	15.1		
		8	7.0	8.2		
		Transitional culture				
		9	14.7	20.2	11.7	19.0
	Near Ryazan'	Ordinary rugose culture				
		1	9.9	17.1		
		2	12.6	15.7		
		3	12.0	22.2		
		Strictly oriented rugose culture				
		4	19.4	30.3		
		5	0.7	0.9		
		Transitional culture				
		6	13.4	23.0		
		7	10.4	20.0		

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TABLE 32 (cont'd)

Zone of Isolation of Culture	Place of Isolation of Culture	Character of Isolated Culture	Quantity of Oxygen Produced by One Billion Cells		Average Values	
			In 5 min	In 10 min	In 5 min	In 10 min
Southern belt	Near Sumy	Strictly oriented rugose culture				
		1	19.5	23.0		
		2	8.6	10.9		
		3	11.1	21.9		
		4	12.5	16.0		
	Near Rostov-on-Don	1	9.4	12.6		
		2	6.2	11.4	9.6	13.5
		3	3.6	5.4		
		4	12.6	15.8		
		5	3.2	5.6		
	Near Krasnodar	1	11.1	16.7		
		2	7.3	9.5		
Dry and moist subtropics	Golodno Step'	Ordinary rugose culture				
		1	14.9	17.0		
		2	11.0	14.4		
		Strictly oriented rugose culture				
		3	2.2	2.6		
		Transitional culture				
		4	6.1	8.4		
		5	4.3	7.6		
	Samarkand		4.4	8.2	7.2	11.7
			4.4	7.4		
			12.0	23.2		
			6.1	11.2		
	Vicinity of Tbilisi	Strictly oriented culture				
		1	7.4	13.8		
		2	3.4	5.6		
		3	4.4	7.4		
		4	6.7	13.7		
		5	14.0	24.0		

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A number of conclusions can easily be drawn from this material. First we note that, in the same soils, there are some cultures with higher catalase activity and some with lower. From the example of a collection taken from the Ryazan soils, it is clear that the catalase activity is not associated with the type of variant of B.mycoides involved. For the very same type, cultures of rather different enzymatic activity can be found.

Despite the deviations of a specialized nature, the decline in catalase activity is very distinct, as one progresses from northern latitudes to southern. Thus, while on the average the northern cultures of B.mycoides produced 20.0 cc oxygen in 5 min per billion cells, only 12.0 cc were produced by the same number of cells of cultures from the middle latitudes, and only 7.0 cc by the southern forms.

Thus, the catalase activity of the races of B.mycoides with an elevated temperature maximum is greatly reduced. Primarily, this can be explained by the fact that microorganisms living at a low temperature would have to develop a more active enzymatic apparatus to maintain their vital functions in a highly depauperate environment.

Secondly, the weakening of the oxidative system may protect southern soil-inhabiting bacteria from high soil temperatures. Oxidative ferments have a low resistance to heat, and organisms rich in them would hardly be viable under environmental conditions where elevated temperatures are the natural occurrence.

This shows that the greater potential for multiplication in the southern cultures is compensated in the northern forms, to some extent, by the more active ferment system.

4. Relation between Climatic Indices and Position of Principal Temperature Points in the Geographical Races of B.Mycoides

No doubt, the temperature conditions of the climate and the growth temperature of soil bacteria are closely interrelated. An analysis of this relationship was of extreme interest, and we attempted to make it.

Table 33 gives certain data on the climatic features of the various localities and the growth temperature of the geographical races of B.mycoides. The same materials are also given in Figs.80 - 82, which show more clearly the essentially regular relationship between the air temperature and the principal growth temperatures of the individual races of B.mycoides.

These results have been taken from the reports of the Moscow Central Meteorological Observatory. It must be noted that, with respect to the soil, the data are highly approximate, since the observation technique of the soil temperature is far from uniform in the various localities. In some localities, the soil temperature is measured beneath vegetation and in some localities in the unplanted areas; this naturally has a great influence on the results. /184

For many places, we obtained no information at all on the soil temperature, for which reason the data for several investigated sites were omitted from the

Tables.

In establishing the relation between the climatic indices and the growth temperature of the geographical races of B.mycoides, it might possibly have been

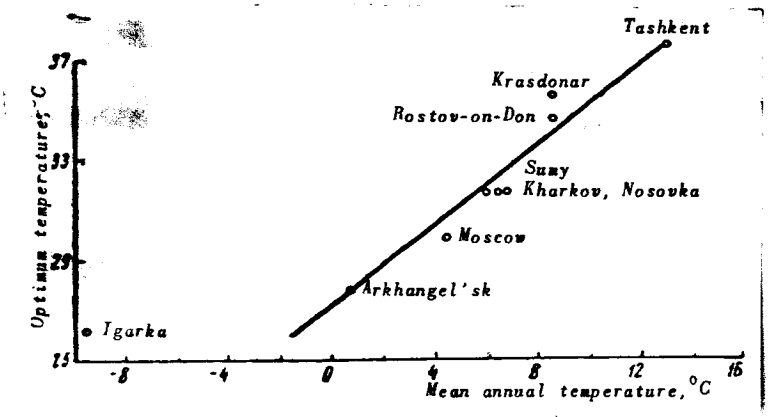

Fig.80 Relation between Optimum Temperature Position for Geographic Races of B.Mycoides and the Mean Annual Temperature

more correct to start from the soil temperature. However, since the soil temperatures are not complete nor sufficiently reliable, we did not consider it possible to use them as a base and instead gave a relation between the ambient

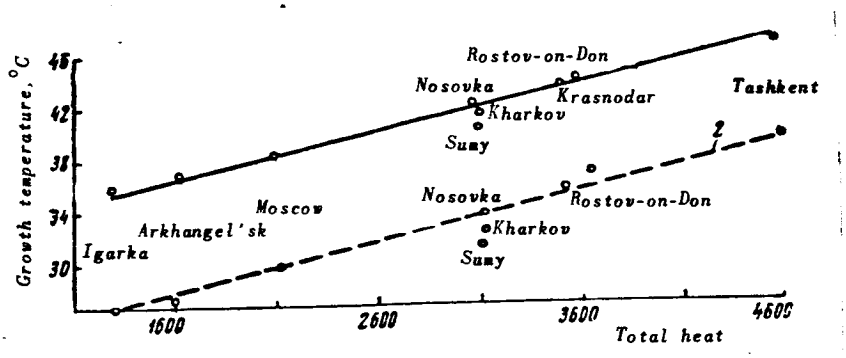

Fig.81 Relation between Optimum and Maximum Temperature Positions for B.Mycoides Races and Total Heat during the Hot Season

temperature and the optimum temperatures, as well as the maximum temperatures of bacterial growth. /185

In our opinion, it is perfectly permissible to connect the latter of these values by a relation, since the heating of the air has a direct influence on the soil temperature.

Further than that, in southern localities, overheating of the soil and its complete drying out is often observed in the summer. Of course, such a temperature cannot be reflected in the adaptive activity of the microbes since it prevents their growth; thus, it is logical that, in the analysis of the causes responsible for adaptation, the heating of the dried-out soil must be disregarded. Obviously, temperature surges of this kind are equalized when the air is heated; at least for the south, this results in a more uniform slope of the temperature curve than the soil.

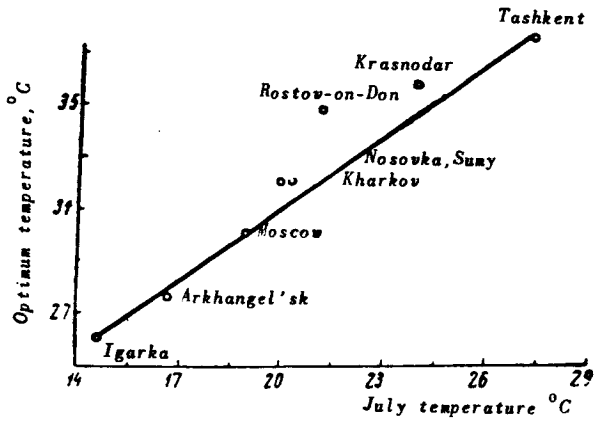

Fig.82 Relation between Position of Optimum Temperature Point for B.Mycoides and Mean July Atmospheric Temperature

For our comparisons, we used the July temperature and the mean annual temperature. In our opinion, the latter is less indicative of the relationship between soil bacteria and temperature than the July temperature, which determines the maximum heating of the soil.

It is particularly difficult to expect a correlation between the mean annual temperature and the principal temperature points of bacteria at localities with a particularly hard winter, such as, for example, at Igarka which is close to the "Cold Pole". It is obvious that here the mean annual temperatures will be far lower, owing to the low temperatures of the winter months, which have no effect on the adaptive activity of the soil bacteria.

Therefore, in setting up the equation of a straight line based on the mean annual temperatures, we used Arkhangel'sk instead of Igarka as the extreme /186
northern point (see below).

The data in Table 33 and the graphic material in Figs.80 - 82 show that a regular relationship exists between the temperature features of the climate and the principal growth temperatures of the bacteria.

It is obvious that this yields a line extremely close to a straight line, where the minor breaks can be neglected. The exceptions shown for the Igarka cultures of B.mycoides are understandable from the above general considerations.

TABLE 33

COMPARISON OF CERTAIN DATA ON THE CLIMATE OF INDIVIDUAL LOCALITIES WITH THE GROWTH TEMPERATURES OF B.MYCOIDES

Location	Atmospheric Temperature (in °C)		Approximate Soil Temperature in July (in °C)	Growth Temperature of Bacteria (in °C)	
	Mean Annual	Mean July		Optimum	Maximum
Arkhangel'sk . . .	0.6	16	about 10	27—28	about 36
Igarka . . .	—10	14.4		about 26	» 36
Moscow . . .	4.2	19	» 19	» 30	»37—38
Kharkov . . .	6.8	21	» 21	» 32	» 41
Sumy . . .	6.1	20	» 21	» 32	» 41
Nosovka . . .	6.4	19.6	» 21	» 32	» 42
Rostov-on-Don . . .	8.9	21	» 23	» 35	» 43
Krasnodar . . .	11.2	24	» 26	» 36	» 44
Tashkent . . .	13.7	27.4		» 38	»45—46

Solution of the straight-line equation for establishing the relation between the mean annual temperature of a given point and the optimum growth temperature of the geographical B.mycoides races yields:

$$\frac{y-27}{38-27}=\frac{x-0.6}{13.7-0.6};\ y=0.84x+26.5.$$

In establishing the relation between the mean July temperature and the optimum growth temperature of the individual B.mycoides races, the following equation can be derived:

$$\frac{y-26}{38-26}=\frac{x-14.4}{27.4-14.4};\ y=0.92x+12.7.$$

Based on the resultant equation, we show in Table 34 results of a theo- /187 retical calculation of the optimum temperature for the various geographical races of B.mycoides. The data indicate that the experimental values are very close to those calculated from the formula. For bacteria isolated in the European portion of the USSR, the agreement is particularly good if the equation derived from the mean annual temperatures is used as basis. These mean temperatures give a more correct idea of the climate than the July temperatures. For example, while the mean annual temperature of Moscow is considerably lower than at Nosovka, the July temperatures of these two points are almost identical. However, Nosovka is obviously isoated in a warmer region. In this case, the mean annual temperature reflects the specific features of the climate more distinctly and thus must be more closely related to the adaptive activity of a given soil bacterium.

Nevertheless, as already noted, this value cannot be adopted without quali-

fication for the extreme northerly points, to establish the desired relationship.

We consider it more correct to compare the principal temperature points of B.mycoides with the sum of the temperatures of the warm days for the summer period (days with temperatures above 10°C). Based on the data of Table 39, the following equation can be derived:

$$\frac{y-26}{38-26}=\frac{x-1350}{4500-1350};\ y=0.0037x+20.9.$$

The number of points used by us for this study was not excessive but was entirely sufficient to establish the existing shifts in the bacterial growth temperatures. Therefore, we believe that the above relations represent a regular phenomenon rather than the result of chance.

TABLE 34

COMPARISON OF EXPERIMENTAL AND CALCULATED OPTIMUM GROWTH TEMPERATURES OF THE GEOGRAPHICAL RACES OF B.MYCOIDES (IN °C)

Points	Temperature (in °C)		Sum of Temperatures for Warm Period	Experimentally Established Optimum Temperature	Calculated Optimum Temperature		
	Mean Annual	Mean July			From Mean Annual Temperature	From July Temperature	From Sum of Temperatures for Hot Season
Arkhangel'sk	0.6	16.0	1600	~ 27.0	27.0	27.2	27.8
Igarka	~ —1.0	14.4	1350	» 26.0	16.1	26	26.0
Moscow	4.2	19.0	2200	» 30.0	30.0	30.2	29.0
Kharkov	6.8	21.0	3100	» 32.0	32.2	32.0	32.4
Sumy	6.1	20.0	2900	» 32.0	31.6	31.1	31.6
Nosovka	6.4	19.2	3000	» 32.0	31.9	30.4	32.0
Rostov-on-Don	8.9	21.0	3400	» 35.0	35.7	32.0	33.5
Krasnodar	11.2	24.0	3500	» 36.0	35.9	32.0	33.5
Tashkent	13.7	27.4	4500	» 38.0	38.0	38.0	38.0

With respect to the maximum temperatures for B.mycoides races, a similar shift takes place with increasing optimum temperature. It might be said that the interval between optimum and maximum temperature for races with an elevated temperature maximum is somewhat narrower than in the more psychrophilic races. For example, the maximum and optimum temperatures for the Arkhangel'sk and Igarka cultures are separated by about 10°C. This interval for the Central Asian races, however, is no more than 8°C.

The available material was insufficient for a categorical statement that

this phenomenon exists, if only for the reason that the optimum temperature /188 does not constitute a point but rather a segment of a straight line, whose exact extent we have not yet experimentally defined. However, there seems to be a tendency to narrowing of the interval between optimum and maximum temperatures in the more thermotolerant races of B.mycoides.

TABLE 35

COMPARISON OF EXPERIMENTAL AND CALCULATED MAXIMUM GROWTH TEMPERATURES OF THE GEOGRAPHICAL RACES OF B.MYCOIDES

Points	Experimental Maximum Temperature	Calculated Temperature	
		From mean Annual Temperature	From Sum of Temperatures of Hot Months
Arkhangel'sk . .	about 36	36.0	36.4
Igarka . .	» 36	28.2	36.0
Moscow . .	» 37—38	38.5	38.2
Kharkov . .	» 41	40.0	41.0
Sumy . .	» 41	39.5	40.0
Nosovka . .	» 42	40.5	40.7
Rostov-on-Don . .	» 43	42.0	41.9
Krasnodar . .	» 44	43.5	42.2
Tashkent . .	» 46	46.0	46.0

As will be seen from Table 35, the mean maximum temperatures of the /189 geographical races of B.mycoides show a rather good dependence on the mean annual atmospheric temperatures (the equation is $y = 0.73x + 35.5$). The only exception is the Igarka race, whose theoretically calculated maximum temperature is considerably below the experimental value. This deviation was entirely natural and has been explained above.

The excellent agreement, as was to be expected, is obtained on comparison of the experimental maximum temperatures and those calculated by an equation set up from the data on the sum of the temperatures for the hot months:

$$\frac{y-35}{46-36}=\frac{x-1350}{4500-1350};\ y=0.0031x+31.4.$$

CHAPTER VI

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UTILIZATION OF THE ADAPTIVE REACTION OF B.MYCOIDES TO CLIMATE FOR AN ANALYSIS OF THE ORIGIN OF GRAIN

After obtaining rather reliable data on the close relation between temperature requirements of B.mycoides and climatic conditions, we desired to utilize this rule for purely practical purposes, namely, for identifying the origin of grain. This question comes up rather often in agricultural practice. Frequently, the use of a given sort of grain from more southerly areas as seed in northern areas, or the reverse practice, results in loss or incomplete maturation of the crops.

Such undesirable consequences can be prevented by proper determination of the point of origin of the grain. This is usually done by analyzing the character of the seeds of the weeds mixed in with the grain. Based on the fact that weeds have a definite area of occurrence, the point of origin of the grain can be determined.

However, this method, although it is relatively simple, has some serious shortcomings.

In the first place, the zone of occurrence of weeds is extremely broad, and it is impossible to accurately define the territory on the basis of their analysis, despite the fact that only the USSR was considered.

Secondly, it is a highly important point that the improvement of the quality of the product, i.e., the cleaning of the grain especially with our transition to large-scale production, can either make such an analysis entirely impossible or in any case considerably hamper it.

Professor Vinner, for example, in his manual "General Agriculture", made the following statements on the determination of the origin of seeds:

"The most difficult task of examination is to determine the place of origin of the seed, since the sample may not always contain the characteristic seeds of the grasses (if the grain seed is well cleaned, weed seeds may be entirely /191 absent) and, in addition, the occurrence of typical weeds can be indicated only very approximately for extensive regions of physical geography, and by far too few localities have been sufficiently studied in this respect".

The seeds of the cultured plants, when grown in different climatic zones, do not acquire specific traits except for certain biochemical properties, which latter may be used as indicators of their origin. This method is restricted, due to certain difficulties connected with the performance of biochemical analysis.

Based on the foregoing, we decided to propose our own method of analysis of

the origin of seed which, if the question cannot be answered by the usual method, may play a large part in agricultural practice. It may also be used to supplement the customary method of analysis.

We started from the assumption that not only seeds of the higher plants, mixed with the grain seed material, but also the bacterial microbes which, according to our studies, bear the stamp of the climate, may serve as indicators in determining the geographical origin of a given shipment of grain.

Soil bacteria of various climatic zones, as already shown, have become so adapted to the temperature conditions of the particular climate that, even if only their maximum and optimum growth temperatures are determined, an accurate idea can be obtained as to the place of origin of any specific soil specimen.

Thus if - as expected - certain species of microorganisms such as soil bacteria, specific for the grain and systematically encountered on such grain will be found to react also to the influence of the climate, then an approach to a solution of the problem could be found. In this case, it most likely will be found that the bacteriological method of grain analysis has various advantages over the customary method.

First, bacteria are never absent from grain; this does away with the objection that the absence of indicator organisms makes an analysis impossible, which latter often is the case when attempting to determine the origin of grain from its weed content.

Second, as our data prove, a study of the growth temperatures of bacteria may yield a more narrow definition of the territorial region than the old /192
method.

Despite the assumed favorable aspects of our method, we must admit that it has some shortcomings.

For example, errors are possible in the analysis of grain from mountain areas, where the bacteria grow at relatively low temperatures. In this case, such seed material might also be thought to come from the northern regions, where temperature has a similar action on the bacteria.

For practical purpose, however, such an error would be negligible since there is very little grain of this kind in the USSR and amounts to only a negligible part of total grain production; secondly, mountain cultures transferred to a more northerly region with a similar climate presumably would also grow successfully there.

The bacterial method will not indicate the continent from which a grain comes, but only its climatic zone of origin. In the case of imported grain, it is sufficient to know the exporting country for answering the question with complete clarity. Certain difficulties that are still unsolved will be mentioned later.

Returning to the method itself, it is of advantage that all necessary operations for analysis of the origin of seed can be performed within a short

time (about three days). The inoculation of bacteria from the material and their germination require about 20 - 24 hours, and the subsequent check of bacterial growth at various temperatures takes not more than 48 hours, provided that the transfers are made directly from the colonies on culture dishes, without first preparing a pure culture.

This enticing prospect makes it logical to envisage the use of "indicator bacteria" which would be easy to isolate from seed material. Of course, it would be necessary to select certain definite bacteria for such work, always of the same species, so as to avoid possible errors due to peculiar bacterial traits. The outer appearance of the species selected must also be so distinct that verification of its identity becomes superfluous.

With these requirements in mind, a check of the epiphytic microflora of grain will show, on the basis of a series of studies, that B.herbicola is the most specific organism.

For this reason, at the beginning of our work in this direction, we attempted to compare the growth temperature of various cultures of this species of bacteria, isolated from grain of various origins.

Several specimens of wheat and rye were taken as the object of study. /193

An abundant number of all specimens of B.herbicola was easily obtained by transfer cultures to meat-peptone agar. From each inoculation, we isolated 3 - 4 cultures of these bacteria which were then tested for growth at various temperatures. For this purpose, a pour culture of meat-peptone agar in Petri dishes, was used for stab cultures of B.herbicola; after two days, we measured the size of the colony.

The results of this study (we omit the corresponding figures) showed that, in contrast to B.mycoides, this bacterium has no marked reaction to the change of climate. Presumably, the medium surrounding the epiphytic microflora varies far less on transition from one climatic zone to another than does the environment in which the soil bacteria live. For this reason, B.herbicola had to be dropped from our list of possible indicator organisms.

Since the typical epiphytic microflora proved unsuitable for our studies, we focused later experiments on the soil microflora which, transferred together with the dust, settles on the surface of the seeds. As already noted, soil bacteria react sharply to the temperature conditions of the climate, making it advisable to utilize this trait for analysis purposes.

Thus, from the number of bacteria systematically found on the grain surface, we had to select some soil bacterium with colonies of characteristic morphological features, and then test our hypotheses on this microbe. We selected B.mycoides which had already been studied by us.

To isolate B.mycoides from the grain, we shook 5 gm grain with 25 cc water in a sterile flask for 6 min. We then transferred 1 cc of the mix to each of several Petri dishes. As will be seen from Table 36, B.mycoides is encountered on grain in constant amounts, although in smaller quantities than other bacteria,

so that its isolation from grain offered no special difficulty.

Since, in culturing bacteria from grain, we frequently found that the Petri dishes become completely covered with sporeless forms of the bacteria, we decided to pasteurize the inoculation material for 5 min at 80°C. On inoculation, this allowed a small number of colonies of spore bacteria to develop, among which B.mycoides also occurred systematically (Table 36).

TABLE 36 /194

FREQUENCY OF OCCURRENCE OF B.MYCOIDES ON GRAIN OF VARIOUS ORIGIN

Place of Origin of Grain	Number of Bacteria per cc of Mix, on Culturing Unpasteurized Material		Total Number of B.Mycoides, on Culturing 1 cc of Pasteurized Mix
	Total Number of Bacteria	B.Mycoides	
Moscow, winter rye	450	1	0
	425	1	1
	370	0	5
	550	2	2
Kharkov, winter wheat	320	1	5
	301	1	6
	280	0	2
	250	1	1
Kharkov, spring wheat	250	1	6
	2[illegible]0	1	0
	350	2	2
	270	1	1
Rostov-on-Don, spring wheat	350	2	4
	450	2	2
	370	0	3
	510	1	1
	590	1	5
	440	3	3
	330	2	2

After we had established that B.mycoides was readily isolated from grain, we verified the growth temperatures of its individual geographic races. These experiments fully confirmed the rules established by us in the preceding Section. A series of successful control tests also confirmed the possibility of using the bacteriological method for determining the origin of grain.

With this method, the optimum growth temperatures of individual races of B.mycoides can be established rather accurately, to within 2°C. It will be clear from Fig.80 that this corresponds to an accuracy of 2° in the mean /195

annual temperature. Within the European portion of the USSR, a 1°C fall in the mean annual atmospheric temperature corresponds to a latitude region as wide as 40 - 100 km in some cases. Thus, the error of indication of our method will hardly exceed an error of latitude of 80 km, or 200 km at most.

Certain auxiliary analyses, such as a determination of the osmotic cell pressure in the geographical races of B.mycoides, will yield an accurate indication as to the longitude of the point of origin of the grain. Factual data on this subject will be given in more detail in later Chapters.

CHAPTER VII

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GENERAL PRINCIPLES OF THE ADAPTIVE REACTION OF SOIL BACTERIA TO THE TEMPERATURE OF THE CLIMATE

1. Position of the Principal Temperature Points for Saprophytic Soil Bacteria

We have already indicated the existence of an adaptive reaction to climatic conditions in the soil bacterium B.mycoides. This was reflected in the variation of the temperature curve of bacterial growth, i.e., in the shift of the minimum, optimum, and maximum temperatures.

There is reason to believe for the adaptive reaction to be a mass phenomenon, obeyed by the multiplicity of soil-inhabiting bacterial species. Of course, individual species may have specific traits affecting the position of the principal temperature points, but the law established for B.mycoides no doubt represents a general trend.

Whereas the object of the above-discussed part of our work was to establish a correlation between the climatic indices and the position of the principal temperature points on the growth curve of B.mycoides, we attempted, in working out the present Section, to demonstrate the general validity of this law.

Thus, the experiments to be described are a logical continuation of a previously started study on the adaptation of soil bacteria to the climatic temperature conditions. We succeeded in accumulating a collection of soils of the European portion of the USSR, running from the Far North (Arkhangel'sk) to the South (Crimea, Caucasus, Central Asia). By isolating a number of bacteria from the soils and applying our usual technique, it was not difficult to establish their principal temperature points.

Table 37 gives a list of the localities from which soils were taken for this work, together with a short climatological characterization of these points.

Taking for granted that adaptation to the climatic temperature conditions in soil bacteria is a mass phenomenon, we did not concentrate our attention /197 on any definite physiological groups of soil bacteria but studied cultures isolated without systematic selection. This arrangement was considered entirely correct, since the law being analyzed, if it existed at all, should be manifest in all groups of the micropopulation of the soil.

Pure cultures of the bacteria were obtained by inoculating the soil on MPA, from which transfers were made after inoculation at 30° C.

The resultant pure cultures were purified by repeated transfer cultures on MPA.

It should be noted that the bacteria used in this work had a primarily

diffuse growth. This facilitated our work with the method adopted.

To check the growth temperature we used the previous method. MPA was poured in a sufficiently thick layer into Petri dishes, and a fresh broth culture of the bacteria was inoculated into it, by a stab with a fine platinum needle. The dishes were then left two days in the incubator at various temperatures, after which the size of the colony was measured by a precision ruler. All experiments were made on two Petri dishes, three stabs on each. The figures in the Table are the mean values of these measurements.

TABLE 37

LIST OF LOCALITIES FROM WHICH SOILS WERE TESTED

Sampling Site (Vicinities)	Latitude (in °C)	Longitude (East of Pulkova)	Mean Annual Atmospheric Temp.	Mean Annual Soil Temperature (20-cm Layer)	July Atmospheric Temperature	July Soil Temperature (20-cm Layer)
Arkhangel'sk	64.5	10.3	0.6	~ 4.1	16	~ 15
Leningrad	59.9	0.0	4.1	» 5.6	18	» 17
Moscow	55.7	7.3	4.2	» 6.3	19	» 18
Kursk	51.7	5.8	5.4	» 8.0	20	» 19
Sinel'nikova	48.3	5.3	9.6	» 9.1	21	» 21
Akapa	44.8	7.0	11.7	—	23	» 26
Armavir	45.0	10.7	9.0	» 11.5	~ 21	» 25
Balaklava	44.6	3.2	12.0	—	23	» 26
Massandra	44.5	3.8	12.7	—	23	» 26
Tashkent	41.0	39.0	13.7	—	27.4	—
Batum	41.5	11.8	14.7	—	24.2	—

In view of the fact that the rate of growth of the individual bacterial /198 species on agar is entirely specific, the data had to be reduced to a common denominator for the further work-up. This was done by taking the size of a colony, obtained at optimum temperature, as 100 and then calculating the size of the colonies grown at other temperatures in relation to it.

The figures for all bacteria of the given soil, obtained at each temperature, were added and the sum was divided by the number of pure cultures required for the work. In this way, we obtained the averages for a given temperature, for the entire set of bacteria studied.

We should mention again that, in speaking of the optimum temperature, we do not understand it as being a point. It has a definite length or range, so that when indicating the optimum temperature, we only approximately fix the central point of this segment of the temperature curve.

We will start the discussion of our results with bacteria of the soils of the northernmost regions.

We obtained our soil specimen from the vicinity of Arkhangel'sk, from which the following soil bacteria were isolated:

1. Motile non-spore-forming rods of genus Achromobacter.
2. Same.
3. Motile non-spore-forming rods of genus Pseudomonas.
4. Same.
5. Same.
6. Motile spore-forming rods, closely resembling B.vulgatus.
7. Same.
8. Motile spore-forming bacillus, closely resembling B.subtilis.

These bacteria were studied by the method described for their behavior toward various temperatures. The data are given in Table 38.

TABLE 38

GROWTH OF BACTERIA FROM ARKHANGEL'SK SOILS AT VARIOUS TEMPERATURES

Bacterial Culture No.	*Size of Bacterial Colonies Related to those Obtained at Optimum Temperatures (in °C)*						
	18	25	29	32	35	39	42
1	7.14	14.3	100	7.1	1.4	0.0	0.0
2	9.09	45.4	100	22.7	12.7	0.0	0.0
3	16.1	38.8	100	46.3	42.6	0.0	0.0
4	6.66	60.0	100	33.3	6.6	0.0	0.0
5	28.6	100	57.1	28.6	22.9	0.0	0.0
6	22.4	52.2	97.0	100	97.0	0.4	0.0
7	62.5	90.3	100	90.3	62.5	0.0	0.0
8	5.9	29.4	100	23.5	9.4	5.9	0.0
Average . . .	19.89	53.7	94.3	43.97	31.9	0.9	—

These figures indicate that most of the bacteria isolated stop growing at 36 - 39° C; their optimum temperature lies near 27 - 30° C. Above 30° C, an appreciable inhibition of growth begins. At 39° C, only two of the eight forms still were growing and that only weakly.

In our study of soils taken from near Leningrad (Detskoye Selo), we isolated the following seven bacterial cultures:

1. Motile non-spore-forming rods of genus Achromobacter.
2. Same.
3. Same.
4. Motile spore-forming rods closely resembling B.vulgatus.
5. Same.

6. Motile non-spore-forming rods of genus Pseudomonas. /199
7. Same.

These bacteria showed the growth temperatures given in Table 39.

TABLE 39

GROWTH OF BACTERIA FROM LENINGRAD SOILS AT VARIOUS TEMPERATURES

Bacterial Culture No.	Size of Bacterial Colonies Related to those Obtained at Optimum Temperatures (in °C)								
	18	25	29	32	35	38	42	46	52
1	12.2	88.9	100	100	88.8	74.4	0.0	—	—
2	10.6	60.0	100	80.0	80.0	70.0	0.0	—	—
3	20.67	47.4	68.4	100	94.7	63.2	0.7	—	—
4	12.4	39.1	34.8	34.8	52.2	100	30.4	21.7	17.4
5	18.6	72.7	100	54.5	36.4	—	0.0	—	—
6	16.5	75.0	76.0	100	50.0	47.5	0.0	—	—
7	18.9	57.1	100	85.7	86.7	47.6	0.0	—	—
Average . .		62.9	82.6	79.3	66.3	57.3	4.4	3.1	2.5

Most of the bacteria from the Leningrad soil have a maximum growth temperature of 40° C. Only one of the series developed above 42° C. The forms of the bacteria with an elevated temperature optimum and maximum, in this case as /200 in all others, were close to B.mesentericus. The optimum temperature for most cultures was near 29 - 31° C.

It should be noted that the virgin podzol soil was taken from the vicinity of Leningrad.

Near Moscow, the bacteria were isolated from the fallow plot "b" of the Sobaka Experimental Station (medium podzol loam) and from the weakly podzolic sandy loam of the Potato Culture Institute at Korenev. The following was the microscopic picture of the bacteria:

I. Bacteria from the Soils of the Sobaka Experimental Station

1. Motile non-spore-forming rods of genus Achromobacter.
2. Same.
3. Motile non-spore-forming rods of genus Pseudomonas.
4. Same.
5. Motile spore-forming bacillus closely resembling B.vulgatus.

II. Bacteria from Soils of the Potato Culture Institute

1. Motile non-spore forming rods of genus Achromobacter.
2. Same.
3. Same.
4. Motile non-spore-forming rods of genus Pseudomonas.
5. Same.
6. Motile spore-forming bacillus closely resembling B.vulgatus.

The testing of these bacteria as to their behavior toward various temperatures yielded the data shown in Tables 40 and 41.

TABLE 40

DEVELOPMENT OF BACTERIA FROM SOIL OF SOBAKA EXPERIMENTAL STATION, AT VARIOUS TEMPERATURES

Bacterial Culture No.	Size of Colonies, Compared to Size at Optimum Temperature (in °C)						
	18	25	29	32	35	39	40
1	14.3	37.5	92.5	100	76.0	37.5	0.0
2	45.0	62.0	100	90.0	71.0	30.0	0.0
3	51.9	61.5	100	38.5	32.7	0.0	0.0
4	20.0	50.0	84.0	100	40.0	32.0	0.0
5	—	62.0	100	64.0	50.0	10.0	0.0
Average	32.8	54.6	91.3	78.5	53.7	21.9	0.0

TABLE 41

DEVELOPMENT OF BACTERIA FROM SOIL OF POTATO CULTURE INSTITUTE, AT VARIOUS TEMPERATURES

Bacterial Culture No.	Size of Colonies, Compared to Size at Optimum Temperature (in °C)						
	18	25	29	32	35	39	42
1	14.3	4.7	79.0	100	51.4	33.3	0.0
2	45	62	100	90.0	70	30.0	0.0
3	37.8	55.0	62.5	100.0	50.0	0.0	0.0
4	36.6	41.5	100	73.1	97.6	0.0	0.0
5	23.8	51.4	66.6	100	61.9	38.0	0.0
6	16.0	66.6	100	53.3	93.3	20.0	0.0
Average	28.9	46.8	84.7	86.1	70.7	20.2	0.0

It is obvious that all these bacteria stop growing at 42° C. The maximum temperature for most of the cultures is close to 40° C, and some of them stop growing at a temperature below 39° C. The optimum for most of these bacteria is in the range of 29 - 31° C.

In general, no great difference was observed between the Moscow and Leningrad bacteria. This was to be expected.

In the chernozem belt, virgin chernozem soil was taken from the vicinity of the City of Kursk.

The following bacteria were isolated:

1. Motile non-spore-forming rods of genus Achromobacter.
2. Motile spore-forming rods closely resembling B.vulgatus.
3. Motile non-spore-forming rods of genus Pseudomonas.
4. Nonmotile non-spore-forming rods of genus Pseudomonas. /201
5. Motile non-spore-forming rods of genus Pseudomonas.

Table 42 gives the relation of these bacteria to various temperatures.

It follows from this material that the optimum temperature of the bacteria in this case is in the range 33 - 35° C, and the maximum around 43 - 45° C.

Thus, by comparison with the Moscow and Leningrad bacteria, we here have a definite shift towards higher temperatures.

TABLE 42 /202

DEVELOPMENT OF BACTERIA FROM SOIL OF THE NEIGHBORHOOD OF THE CITY OF KURSK

Bacterial Culture No.	Size of Colonies, Compared to Size at Optimum Temperature (in °C)								
	18	25	29	32	35	39	42	46	50
1	13.3	20.0	73.3	74.7	100	1.3	0.0	0.0	0.0
2	3.1	68.7	93.7	93.7	100	46.0	3.1	0.0	0.0
3	41.2	82.3	91.8	98.4	100	84.3	76.5	0.0	0.0
4	18.1	60.2	84.3	100	92.8	96.4	18.1	0.0	0.0
5	25.6	38.5	79.5	88.2	82.1	76.9	83.5	0.0	0.0
Average	20.3	53.9	84.5	89.1	95.2	64.6	36.2	0.0	0.0

Chernozem of the Ukraine was taken from a plowed field in the vicinity of the City of Sinel'nikovo (Kharkov Okrug). A brief survey of the seven cultures of bacilli taken from this soil yields the following picture:

1. Motile non-spore-forming bacillus of genus Achromobacter.
2. Same.
3. Same.
4. Immotile non-spore-forming bacillus of genus Achromobacter.
5. Motile spore-forming rods closely resembling B.vulgatus.
6. Same.
7. Motile non-spore-forming rods of genus Pseudomonas.

Table 43 shows the growth of these bacteria at various temperatures.

TABLE 43

DEVELOPMENT OF BACTERIA FROM SOIL OF SINEL'NIKOVO STATION

Bacterial Culture No.	Size of Colonies, Compared to Size at Optimum Temperature (in °C)							
	18	25	29	32	36	38	42	46
1	29.4	—	41.2	29.4	68.0	100.0	94.4	19.6
2	40.5	92.8	97.6	100	71.4	38.1	0.0	0.0
3	47.6	40.7	71.4	83.3	100.0	95.2	0.0	0.0
4	27.3	74.5	63.6	90.9	100.0	96.3	90.9	0.0
5	21.1	46.7	88.9	91.1	100.0	91.1	84.4	0.0
6	31.2	49.9	65.6	—	78.1	100.0	93.7	0.0
7	30.76	73.0	48.1	77.1	100.0	96.1	67.2	0.0
Average	35.2	63.8	68.1	78.6	91.1	88.2	61.5	2.8

By comparison with the preceding Kursk bacteria, the Ukrainian types /203
grow somewhat better at elevated temperatures (38 and 42°C). There is also a certain shift of the optimum toward higher temperatures. This is well defined in the range of 34 - 37°C. The maximum for most bacteria lies in the range 43 - 45°C. Two species of bacteria, however, stopped growth below 42°C.

From the North Caucasian Kray, two chernozem soils were taken for this work. The more northerly virgin chernozem was taken from the vicinity of the City of Armavir and the more southerly specimen, from a cultivated field near the City of Anapa.

We isolated the following bacteria from these soils:

1. Motile non-spore-forming rods of genus Achromobacter.
2. Same.
3. Motile spore-forming rods closely resembling B.vulgatus.
4. Motile non-spore forming rods of genus Pseudomonas.
5. Same.
6. Nonmotile non-spore-forming rods of genus Pseudomonas.
7. Same.

8. Motile non-spore-forming rods of genus Pseudomonas.

From Soils of City of Anapa:

1. Motile non-spore forming rods of genus Achromobacter.
2. Nonmotile non-spore-forming rods of genus Achromobacter.
3. Nonmotile non-spore-forming rods of genus Pseudomonas.
4. Nonmotile non-spore-forming rods of genus Pseudomonas.
5. Motile non-spore-forming rods closely resembling B.vulgatus.

Tables 44 and 45 show the growth of these bacteria at various temperatures.

The growth rate curve for the Armavir bacteria closely resembles that for the Sinel'nikova bacteria. The optimum here is likewise at 35 - 37° C, and most of the bacteria still grow above 42° C. Their maximum temperature evidently is close to 44 - 45° C.

TABLE 44

DEVELOPMENT OF BACTERIA IN SOILS FROM THE VICINITY OF THE CITY OF ARMAVIR

Bacterial Culture No.	Size of Colonies, Compared to Size at Optimum Temperature (in °C)								
	18	25	29	32	35	38	42	46	52
1	17.2	20.7	44.8	62.0	100	89.6	27.6	0.0	—
2	37.5	50.0	52.5	87.5	100	87.5	50.0	0.0	—
3	38.5	46.1	64.6	70.8	100	92.3	84.6	33.8	50.8
4	32.0	40.0	24.0	75.0	100	80.0	49.0	0.0	—
5	35.7	57.1	72.8	35.7	100	78.6	64.3	0.0	—
6	88.1	8.1	48.4	51.6	64.5	100	96.8	72.6	24.9
7	17.6	23.5	36.5	37.6	100	76.5	49.4	0.0	—
8	20.0	30.0	44.0	84.0	100	96.0	90.0	0.0	—
Average	25.8	35.7	48.5	63.0	95.6	87.6	62.8	13.3	9.4

However, a study of the growth temperatures of the Anapa cultures clearly shows an increase of the optimum to 38 - 39° C. The maximum temperature also appears somewhat higher for most of these bacteria than for the Armavir bacteria (this could be fixed as being between 46 and 47° C).

We used two soils from the Crimean Peninsula. One of these, taken from a field under wheat near Balaklava, was a chernozem while the other was a slaty soil from a vineyard at Massandra.

Below, we will give a short description of the bacteria isolated.

TABLE 45 /204

DEVELOPMENT OF BACTERIA IN SOILS FROM THE VICINITY OF THE CITY OF ANAPA

Bacterial Culture No.	Size of Colonies, Compared to Size at Optimum Temperature (in °C)								
	18	25	29	32	35	38	42	46	50
1	14.3	35.7	50.0	92.0	100	92.9	60.0	0.0	0.0
2	8.7	13.0	34.8	39.1	56.5	100	73.9	36.9	0.0
3	13.1	65.8	—	57.9	65.8	100	97.4	0.0	0.0
4	13.3	33.3	73.3	80.0	100	86.7	73.3	26.7	0.0
5	26.1	69.2	92.3	84.6	92.3	100	98.5	100	0.0
Average	15.1	43.4	62.6	70.9	82.9	95.9	69.2	12.7	0.0

From Soils of City of Balaklava:

1. Motile spore-forming rods closely resembling B.vulgatus.
2. Motile non-spore-forming rods of genus Achromobacter.
3. Nonmotile spore-forming rods of genus Achromobacter.
4. Motile spore-forming rods closely resembling B.subtilis.
5. Motile non-spore-forming rods of genus Pseudomonas.
6. Same.
7. Same.
8. Same.
9. Nonmotile non-spore-forming rods of genus Pseudomonas. /205
10. Same.

Bacteria from Soils of Village of Massandra:

1. Motile non-spore-forming rods of genus Achromobacter.
2. Nonmotile non-spore-forming rods of genus Achromobacter.
3. Nonmotile non-spore forming rods of genus Achromobacter.
4. Motile spore-forming rods closely resembling B.vulgatus.
5. Motile spore-forming rods closely resembling B.subtilis.
6. Motile non-spore-forming rods of genus Pseudomonas.
7. Same.
8. Nonmotile non-spore-forming rods of genus Pseudomonas.

Tables 46 and 47 show the character of growth of these bacteria.

It follows from our data that the optimum temperature of the Crimean bacteria is about 36 - 39° C, and that the maximum lies near 44 - 45° C.

Near the City of Batum, we obtained a specimen of krasnozem from which the following bacteria were isolated:

1. Motile non-spore-forming rods of genus Pseudomonas.
2. Same.
3. Same.
4. Same, of genus Achromobacter.
5. Same.
6. Same.

TABLE 46

DEVELOPMENT OF BACTERIA FROM SOILS OF CRIMEA (BALAKIAVA) AT VARIOUS TEMPERATURES

Bacterial Culture No.	Size of Colonies Related to Size at Optimum Temperature (in °C)							
	18	25	29	32	36	39	42	46
1	16.4	29.5	26.2	32.8	98.4	100.0	81.4	0.0
2	29.7	27.0	32.4	94.6	93.3	100.0	67.6	0.0
3	5.3	26.3	31.6	62.6	63.3	100.0	47.4	0.0
4	20.0	24.0	30.0	80.0	76.0	100.0	98.0	60.0
5	30.0	40.0	50.0	42.0	100.0	60.0	60.0	56.0
6	22.2	23.3	37.8	40.0	—	100.0	55.6	0.0
7	5.1	8.5	16.9	37.3	100.0	79.7	67.8	0.0
8	14.3	20.0	28.6	42.9	100.0	84.3	78.6	0.0
9	14.1	23.4	15.6	43.7	93.7	100.0	54.7	0.0
10	25.9	37.0	38.9	46.3	74.1	100.0	92.6	0.0
Average	18.3	26.9	30.8	52.2	89.4	93.0	71.4	11.6

TABLE 47

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DEVELOPMENT OF BACTERIA IN SOILS FROM THE CITY OF MASSANDRA, AT VARIOUS TEMPERATURES

Bacterial Culture No.	Size of Colonies, Compared to Size at Optimum Temperature (in °C)								
	18	25	29	32	35	39	42	46	52
1	42.2	62.2	77.8	87.0	100.0	66.7	66.7	0.0	—
2	53.3	64.4	64.4	100.0	87.0	77.5	0.0	0.0	—
3	46.1	58.5	60.8	90.8	90.8	100.0	92.3	0.0	—
4	14.3	20.0	23.8	75.2	49.5	100.0	85.7	0.0	—
5	12.7	26.7	30.0	34.0	100.0	93.3	61.3	0.0	—
6	15.6	31.2	37.8	62.5	65.6	100.0	75.0	0.0	—
7	20.8	19.2	28.5	50.0	100.0	69.2	46.1	0.0	—
8	34.5	67.2	70.7	86.2	98.3	100.0	94.8	69.3	51.7
Average	29.9	43.7	49.2	73.2	85.8	88.4	65.2	7.5	6.5

7. Same.
8. Motile spore-forming rods closely resembling B.subtilis.
9. Same.
10. Motile spore-forming rods closely resembling B.vulgatus.

These bacteria were studied as to their behavior toward temperature. Table 48 gives data, permitting the conclusion that the optimum temperature for the bacteria of the Batum soils is close to 37 - 39° C, and that their maximum temperature is near 53 - 54° C.

From soils of Golodno Step' (Central Asia) under alfalfa, we isolated and studied the following bacteria:

1. Motile non-spore-forming rods of genus Achromobacter.
2. Same.
3. Same.
4. Motile non-spore-bearing rods of genus Pseudomonas.
5. Motile spore-forming rods closely resembling B.subtilis.
6. Motile spore-forming rods closely resembling B.vulgatus.
7. Same.
8. Same.
9. Motile spore-forming rods closely resembling B.subtilis.
10. Same.

Table 49 gives the data on the growth of these bacteria at various temperatures.

TABLE 48

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DEVELOPMENT OF BACTERIA IN SOILS FROM THE VICINITY OF THE CITY OF BATUM, AT VARIOUS TEMPERATURES

Bacterial Culture No.	Size of Colonies, Compared to Size at Optimum Temperature (in °C)						
	18	30	36	39	42	52	56
1	10	69	80	100	70	15	0
2	27	54	100	97	74	48	0
3	15	68	88	100	50	18	0
4	28	87	95	100	85	20	0
5	14	80	100	100	97	57	0
6	15	55	90	100	75	30	0
7	10	60	100	25	44	40	0
8	9	85	95	100	65	80	0
9	0	45	91	100	68	57	20
10	7	58	87	100	71	40	0
Average	13.5	66.1	92.6	98.2	69.9	40.5	0.2

TABLE 49

DEVELOPMENT OF BACTERIA IN SOILS FROM THE VICINITY OF GOLODNO STEP' AT VARIOUS TEMPERATURES

Bacterial Culture No.	Size of Colonies, Compared to Size at Optimum Temperature (in °C)								
	18	25	28	30	36	39	42	50	56
1	9.0	16	39	48	91	100	48	0.0	0.0
2	7.0	21	38	68	100	79	10	0.0	0.0
3	12	30	33.0	71	100	62	53	21	0.0
4	7	12.5	25.0	30.0	100	100	75	40	0.0
5	11	32	38	46	91	100	42	15	0.0
6	14	38	63	75	88	100	95	30	0.0
7	8	20	30	70	80	100	50	48	0.0
8	11	28	37	73	91	100	90	37	0.0
9	5	14	26	45	100	100	72	44	0.0
10	10	15	17	51	84	100	77	33	0.0
Average	9.4	22.6	34.6	56.6	92.5	94.1	61.2	26.7	0.0

The data in Table 49 show that the optimum temperature for most of the saprophytic bacteria of the Golodno Step' soils is within the range of 37 - 39° C and the maximum is over 50° C (evidently near 51 - 52° C).

The above data give a fairly regular picture of the rise in optimum and /208 maximum temperatures for soil bacteria, as one progresses from the northern regions to the more southerly regions. However, considering the lower temperature end (18° C), no clearly defined indications of intensified growth in the northern bacteria can be discovered.

This can be explained primarily by the fact that the technique adopted by us gives satisfactory results only if the size of the colonies on Petri dishes is sufficient to be measured readily. Since we had to make all our temperature experiments simultaneously, a short time after inoculation, it is logical that the colonies obtained at a low temperature were very small in size. The error in their measurement could have been substantial, which would explain the inaccuracy of the results.

However, it seemed of interest to compare the growth of bacteria of various climatic zones rather than only at elevated temperatures. Therefore, we made the following comparative experiment, using a count of bacteria grown in a liquid medium (meat-peptone broth) within a specified time (two days). These data were obtained for the optimum and the investigated temperatures, yielding a numerical index of rate of growth for the temperatures studied. Several bacteria were studied in this way. The data are given in Table 50.

This experiment definitely shows that, at a temperature of 18° C, the northern bacteria developed relatively faster than the southern. There is reason to

believe that, at still lower temperatures, this difference will be even more distinct.

From a typical analysis of a series of more specific physiological groups of bacteria we attempted to confirm the general existence of an adaptive reaction

TABLE 50

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GROWTH OF BACTERIA FROM VARIOUS SOILS AT 18° C

Bacterial Culture No.		Number of Bacteria in 1 cc of Medium (in millions)		Ratio of II : I (in %)	Average
		Growth at Optimum Temperature	Growth at 18°C		
		I	II		
Arkhangel'sk	1	32.4	15.2	46.9	
	2	24.4	25.6	104.9	
	3	37.2	35.6	94.1	
	4	40.0	28.0	70.0	64.53
	5	63.2	41.6	65.8	
	6	33.6	18.4	54.8	
	7	76.8	60.8	73.6	
	8	53.6	35.2	65.7	
Leningrad	1	36.0	24.0	66.6	
	2	44.0	32.0	72.7	
	3	72.0	36.0	50.0	61.3
	4	60.0	36.0	60.0	
	5	56.0	32.0	57.4	
Moscow (Korenevo)	1	80.0	48.0	60.0	
	2	56.0	24.0	42.8	
	3	52.0	28.0	53.8	54.7
	4	60.0	36.0	60.0	
	5	56.0	32.0	57.1	
Kursk	2	34.8	5.2	14.2	
	2	53.6	16.8	31.38	20.73
	3	45.2	10.8	23.9	
	4	18.8	2.4	12.8	
Anapa	1	39.2	4.0	10.2	
	2	27.6	8.4	30.4	
	3	26.8	4.4	16.4	20.3
	4	22.0	8.0	34.5	
	5	24.0	2.4	10.0	

of soil microorganisms to the temperature conditions of the climate. For this purpose, we isolated the following groups of bacteria from various soils:

1. Denitrifying bacteria.
2. Urea (urea decomposers).
3. Gelatinolytic bacteria.
4. Nitrobacter.

Pure cultures were isolated from the enriched cultures obtained after several successive transfer cultures.

The temperature relations of the denitrifying bacteria were determined in the following manner: Test tubes filled with a high layer of conventional /209
Giltay's medium were inoculated with the bacterial cultures and placed in the incubator at various temperatures. We noted the time required for complete disappearance of the reaction for nitrates and nitrites. Table 51 gives the results.

As shown by these data, the denitrifiers of Moscow soils stop their growth at 42° C, the bacteria from Crimean soils at 46° C and those from Batum soils at 50° C. At low temperatures, the northern forms are more active.

TABLE 51 /210

GROWTH OF DENITRIFYING BACTERIA AT VARIOUS TEMPERATURES

Bacteria Investigated	*Time for Disappearance of Nitrates, in Days, at Temperature (in °C)*				
	20	35	42	46	50
1	2	3	4	5	6
From Moscow soils:					
Culture 1	3	2	Traces of process	No process	
2	3	2	Same	Same	
3	2.5	2	-	-	
4	3	2	-	-	
5	3	2	-	-	
6	3.5	2	-	-	
From soils of the south Crimean coast:					
Culture 1	4	2	2.5	No process	
2	4	2	3	Same	
3	4	2	2.5		
4	3.5	2	2	-	
5	4	2	3	-	
6	3.5	2	2.5	-	
From soils near Batum:					
Culture 1	4.5	2	2	3	Traces of process
2	4	2	2	3.5	
3	4.5	2	2	3.5	-
4	4.5	2	2	3	-
5	4	2	2	3.5	-

In this experiment, we took a relatively small number of temperature points, which precluded a very accurate determination of the optimum. There is no doubt, however, that a shift toward higher temperatures can be observed in the southern forms of the bacteria.

In general, the behavior of the denitrifiers follows the law already noted

for the geographical races of B.mycoides and other saprophytic soil bacteria.

An identical picture is obtained in the study of urea-decomposing bacteria. The maximum temperature here is very close to the temperatures of the other groups of mesophilic bacteria studied by us (Table 52).

The group of gelatinolytic bacteria was studied by the giant-colony method to define their temperature relations. Table 53 gives the results. The maximum size of a colony of the bacteria, grown under the most favorable conditions, is taken as 100. The values given are the averages of four measurements. /211

TABLE 52

GROWTH OF UREA BACTERIA AT VARIOUS TEMPERATURES

Bacteria Investigated	Quantity of NH_3 in mg, Formed in 10 cc of Medium with Urea in 2 Days at Temperature (in °C)					
	20	26	33	45	50	55
From Moscow soil:						
Culture 1	1.2	18.2	15.4	0.0	—	—
2	1.4	17.9	15.5	0.0	—	—
3	1.7	18.2	16.4	0.0	—	—
4	1.2	16.1	18.2	0.0	—	—
5	1.4	13.4	18.5	0.0	—	—
From Crimean soil:						
Culture 1	0.0	13.7	14.0	14.3	0.0	—
2	1.7	17.9	19.2	13.7	0.0	—
3	0.0	13.8	17.4	12.0	0.0	—
4	0.0	1.37	14.4	10.2	0.0	—
5	0.3	1.51	17.4	12.4	0.0	—
From Central Asian soil:						
Culture 1	0.0	8.5	19.4	16.3	8.6	0.0
2	0.0	14.3	18.2	14.8	traces	0.0
3	0.0	13.7	19.7	17.1		0.0
4	0.0	15.2	20.3	25.3	10.1	0.0
5	0.0	12.3	16.8	15.1	9.8	0.0

Note. The medium contained 0.5% of urea.

It is difficult, from the data of Table 53, to draw any conclusion as to the optimum temperature of these bacteria, since the temperature intervals were very great. However, the position of the maximum temperature points is rather well outlined.

It will be seen that most of the microflora of the coldest places of the mountainous Crimea and the Moscow Oblast stops growing at 40° C, and the more southerly soils of the Crimea contain microflora with a maximum temperature up to 43 - 45° C, while the maximum temperature of the Central Asian forms is still higher, reaching 50 - 55° C.

As for the minimum temperatures, we observe the opposite relation, for

TABLE 53

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GROWTH OF GELATINOLYTIC BACTERIA AT VARIOUS TEMPERATURES

Bacteria Investigated	*Size of Bacterial Colonies Related to that Obtained at Optimum Temperatures (in °C)*							
	15	18	26	35	40	45	52	56
In Moscow soils:								
Culture 1	0.0	44.1	88.2	100	73.5	0.0	—	—
2	5.5	41.6	94.4	100	0.0	0.0	—	—
3	0.0	57.1	100	92.8	0.0	0.0	—	—
4	8.3	25.0	94.4	100	0.0	0.0	—	—
5	38.4	46.1	100	92.3	0.0	0.0	—	—
6	11.4	86.6	73.2	100	0.0	0.0	—	—
Average	10.6	50.1	91.7	99.2	12.2	0.0	—	—
In Crimean soils:								
Culture 1	0	30.8	62.5	100	83.3	58.3	0.0	—
2	0	11.4	80.0	100	83.4	0.0	0.0	—
3	0	19.2	61.5	85.3	100	0.0	0.0	—
4	4.6	43.3	80.0	93.3	100	10.0	0.0	—
5	41.7	66.6	87.1	100	70.8	0.0	0.0	—
6	18.7	62.5	100	87.5	83.3	0.0	0.0	—
Average	10.9	38.9	78.5	94.3	88.4	11.4	0.0	—
From soils of Crimean mountains:								
Culture 1	21.1	25.9	83.7	100	0.0	0.0	—	—
2	17.8	44.4	81.8	100	0.0	0.0	—	—
3	24.4	40.0	82.2	100	0.0	0.0	—	—
4	44.3	62.5	94.3	100	75.4	0.0	—	—
5	37.1	81.8	90.9	100	0.0	0.0	—	—
Average	29.9	51.1	86.6	100	15.1	0.0	—	—
From soils of Central Asia:								
Culture 1	0.0	—	40.4	83.1	100	35.4	24.3	0.0
2	0.0	—	68.0	92.5	100	43.0	15.0	0.0
3	7.0	—	53.2	100	90	76.3	7.0	0.0
4	0.0	—	68.8	74.3	100	54.0	30.1	0.0
5	9.0	—	94.2	89.1	100	68.5	14.3	0.0
6	11.0	—	48.4	95.4	100	55.3	9.8	0.0
Average	4.5	—	50.5	89.1	98.6	55.4	16.7	0.0

example, at 15° C the Moscow and the mountainous Crimean flora grow better than the southern flora. The difference is particularly clear if the various cultures are made by stabs into a gelatin column. The soil bacteria of the northern zone give a rapid and vigorous liquefaction, in which they differ markedly from the southern flora, which are rather slow to peptonize gelatin. From the /213 appearance of the column, we were able to accurately separate the bacteria of northern and southern soils.

Thus, in the most widespread mesophilic flora of the soil, we observed a distinct shift in growth temperatures, which was of the nature of an adaptation to climate. It is true that each of the cultures studied has its own characteristic difference, but the general law is clearly delineated. It is interesting that we isolated bacteria from the Moscow and Crimean soils both in summer and winter (January), but noted no differences in the temperature relations. This suggests constancy of the differences. In our observations, we never encountered, during the hot season of the year, any sizeable amount of individuals able to tolerate higher temperatures.

Azotobacters showed a peculiar picture in their temperature relation. Yenikeyeva, of our laboratory staff, isolated cultures of azotobacter (Azotobacter chroococcum) from several highly contrasting soils. These bacterial cultures were then checked by our usual method, as to their temperature relations. As indicated in Table 54, all cultures studied, regardless of origin, grew in about the same temperature range. Their growth always stopped at 40 - 41° C.

TABLE 54

RELATION OF VARIOUS CULTURES OF AZOTOBACTER CHROOCOCCUM TO TEMPERATURE

Soil from which Azotobacters were Isolated	Growth of Culture of Azotobacters at Temperature (in °C)						Principal Temperature Points of Saprophytic Bacteria	
	15	26	35	37	40—41	42—43	Optimum	Maximum
Moscow, podzol	+	++	++	+++	—	—	30	40—42
Same	+	+++	+++	++	traces	—	—	—
Rostov-on-Don, chernozem	++	+++	+++	++	traces	—	34—36	43—45
Frunze, serozem	+	++	++	+++	traces	—	—	—
Samarkand, serozem	+	+++	+++	+++	—	—	38	56—54

Note. + detectable growth; ++ satisfactory; +++ good growth.

Harder reached substantially similar conclusions from his work. In his studies, the southern cultures of azotobacters had a slightly elevated maximum temperature.

Azotobacter in general makes rather specific demands on its environment. It is considerably more hydrophylic than most saprophytic soil bacteria. This, among other things, makes us agree with the view of several other authors that it is a water organism. But a denizen of a watery environment, for obvious reasons, should have more uniform requirements than soil microorganisms. Obviously, the azotobacter is a relatively recent inhabitant of the soil, and has not yet succeeded sufficiently in acclimating itself to the specific character of soil conditions.

In fact, recent studies by Imshenetskiy (1946) on the phylogenesis of azotobacters confirm its relation with the class of Cyanophyceae.

In the above experimental material, we had occasion to note the absence of any pronounced adaptive reaction in other, non-soil-inhabiting, bacteria. For example, so typical an epiphytic bacterium as B.herbicola did not appreciably change its growth temperatures on transition from the northern zone to the /214 southern.

2. Demonstration of Environmental Adaptation of Bacteria by the Method of Microbiological Reactions

The general existence of an adaptive reaction to the temperature conditions of a given climate is easily demonstrated by use of the Remi method of microbiological reactions. If elective nutrient media are inoculated with known weighed portions of soil, it can be shown that, at low temperatures, the microbiological processes proceed faster in the vessels inoculated with northern

TABLE 55

DENITRIFICATION RATE AFTER INOCULATION OF MEDIUM WITH VARIOUS SOILS

Soil Investigated	*Rate of Process in Days, at Temperature (in °C)*					*Remarks*
	18	25	30	35	40	
Moscow, Botanical Garden	3.5	2.5	—	2	2	
Moscow, plowed field of Timiryazev Agric. Academy	3.0	2.5	—	2	2	
South Coast of Crimea, vineyard . . .	4.5	3.0	—	3.0	2	
South Coast of Crimea, tobacco crop . . .	4.5	3.5	—	2.5	2	
Mountainous Crimea, virgin soil of Yayla . . .	3.0	2.5	—	2	2	
Vicinity of Batum, Botanical Garden . . .	5.5	—	3	—	6	
Vicinity of Batum, tea plantation . . .	5,5	—	3.5	—	5.5	

soils. It is obvious that the bacteria of northern soils are more adapted to low temperatures. This is also manifested in the rate and vigor of some specific processes. At higher temperatures (35 - 40° C), the differences between the individual soils are obliterated, since such conditions are rather favorable for the mesophilic and thermophilic (thermotolerant) microflora of both northern and southern forms of bacteria.

We studied a number of the following processes by the Remi method.

The rate of denitrification was checked on Giltay's medium, which was poured into test tubes in a high layer. Into each test tube, 0.5 gm of soil was introduced. Pairs of test tubes were prepared for the various temperatures, /215 noting the formation of gas and the accumulation or disappearance of nitrites. Table 55 gives data obtained in these experiments. The vertical columns of this Table show the number of days required for the complete disappearance of the nitrates and nitrites.

In studying denitrification, a striking fact is the rapid increase of nitrites in the samples from northern and mountainous soils at 18° C, and in part at 25° C, already in the first stage. In southern soils, the process is slow at these temperatures. High temperatures give no appreciable difference for all soils except for those of Batum, which are poor in thermophilic microflora (see below).

The rate of proliferation of urea bacteria was studied in a 10% solution of urea. The urea was dissolved in a medium containing 0.5% Liebig meat extract and 0.1% K_2HPO_4. The medium was prepared with tapwater.

The urea was filtered through a Chamberland candle and added to the nutrient solution, 50 cc of which was poured into several small Erlenmeyer flasks each. The sterile media were inoculated with 0.5 gm of the test soil. Under the action of the urea bacteria in the soil, the urea began to decompose, alkalizing the medium. The rate of decomposition of the urea could be judged from the degree of alkalization of the nutrient medium. This was determined by titrating, at definite intervals, a certain quantity of the medium with 0.1 N sulfuric acid, using rosolic acid as indicator.

Table 56 gives the data characterizing the decomposition of urea by /216 urea bacteria from various soils.

In this experiment, the data for the second day were more significant. Later, the urea decomposition proceeds to completion, so that the difference between the individual flasks evens out.

In general, as in the preceding experiments, the microflora of the soils of colder climatic regions (Moscow, mountainous parts of the Crimea) is easier to grow at 18° C. This suggests that it is more adapted to existence at a low temperature.

The rate of ammonification was studied in a 1% peptone solution in tapwater to which 0.1% K_2HPO_4 and 0.1% NaCl were added. A total of 100 cc sterile medium was poured into each Erlenmeyer flask, and after sterilization each flask was

inoculated with 1 gm of the test soil. After two days, from all flasks at various temperatures, the ammonia was driven off and determined by titration with 10 N H_2SO_4.

TABLE 56

DECOMPOSITION OF UREA ON INOCULATION OF THE MEDIUM WITH VARIOUS SOILS

Soil Investigated	Quantity of NH_3, in mg per 100 cc Medium, Formed at Temperature (in °C)							
	In 2 days				In 4 Days			
	18	25	35	40	18	25	35	40
Moscow, Botanical Garden	1598	1940	1836	1938	2227	2210	1938	1972
Moscow, plowed field, Timiryazev Agric. Acad.	1462	1968	1870	1734	—	—	—	—
South Coast of Crimea, Vineyard	136	1207	2210	2193	1670	2122	2261	2159
South Coast of Crimea, tobacco crop	102	1717	2254	2193	1632	2278	2244	2295
Mountainous Crimea, virgin soil of Yayla	1530	1887	2040	1904	—	—	—	—
Vicinity of Batum, Botanical Garden	85	1326	1309	—	—	—	—	—
Vicinity of Batum, tea plantation	78	1541	1766	—	—	—	—	—

Table 57 gives the quantity of ammonia in mg formed in 100 cc of nutrient medium. The averages of two parallel determinations are given. In general, these determinations were very close together.

TABLE 57

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DECOMPOSITION OF PEPTONE ON INOCULATION OF THE MEDIUM WITH VARIOUS SOILS

Soil	Quantity of NH_3 in mg per 100 cc Medium Formed at Temperature (in °C)			
	18	25	35	40
Moscow, Botanical Garden	165.0	205.2	196.5	198.0
Moscow, field of Timiryazev Agricultural Acad.	136.9	165.6	186.0	180.5
South Coast of Crimea, Vineyard	52.8	109.6	128.4	180.6
South Coast of Crimea, tobacco field	36.3	112.5	192.6	193.2
Vicinity of Batum, Botanical Garden	37.5	30° C 175.1		135.3

Here again, we note that the energy of the bacterial process is greater at 18 and 25° C when the medium is inoculated with the microflora from Moscow soils. The bacteria of the southern coast of the Crimea and the vicinity of Batum, at the same temperatures, decomposed peptone very slowly.

A temperature of 35 and 40° C, for all soil specimens, gave almost the same rate of ammonification.

Nitrification was studied in Vinogradskiy's medium with $(NH_4)_2SO_4$. A total of 30 cc of this nutrient solution was poured into each of a series of small Erlenmeyer flasks, so that the liquid formed a thin layer of about 1.5 cm on the bottom. Then, 0.5 gm of soil was added to the flask. Two flasks were inoculated with the same soil at each temperature. All flasks were analyzed daily for appearance of a distinct reaction for nitrates and nitrites. Table 58 gives the number of days required from the moment of inoculation to the beginning of formation of HNO_2 and HNO_3. The process of nitrification was studied only for the soils from the Moscow Botanical Garden, the Crimea (from a vineyard) and the Batum Botanical Garden.

As shown, the result of the previous experiments is repeated on this example.

TABLE 58

NITRIFICATION ON INOCULATION OF A MEDIUM WITH VARIOUS SOILS

Soil	Time to Appearance of Reactions, in Days, at Temperature (in °C)				Remarks
	18	25	35	40	
	Incipient HNO_3 formation				
Moscow	5	4	3	1	Distinct reaction with brucine
Crimea	7	5	3	1	
Batum	8	5	3	1	
	Incipient HNO_2 formation				
Moscow	3	3	2	1	Distinct reaction with Griess' reagent
Crimea	6	5	2	—	
Batum	7	4	2	—	

Our work on nitrifying bacteria was later completely confirmed by Thandon and Dhar.

Thus, an examination of these Tables yields a clear law which repeats in each of the processes. This law states that the bacterial population of soils from regions with a cold climate has adapted to life at lower temperatures,

which are no longer favorable for the microflora of regions with a mild climate. Here, we observe a temperature shift of the process in one direction or the /218 other, depending on the conditions under which the population had been living in its natural habitat.

In conclusion, it should be noted that the same soil specimens were investigated by us during various seasons (summer, autumn, and winter). These repeated experiments fully confirmed the correctness of our observation that the northern microflora was always better adapted to a lower temperature than the southern microflora.

3. Psychrophilic and Thermophilic Soil Microflora

The mesophilic soil microflora, with which we were working at temperatures from 15 to 55° C, grow very little at the low temperatures encountered in the soil during the cold time of the year. Some investigators, such as Conn, considered that there is a certain rearrangement of the soil microflora in the autumn and winter period. Conn noted the appearance at this time of a considerable number of bacteria giving small punctiform colonies, when cultured on nutrient media. He considered these microorganisms to belong to the psychrophilic microflora of the soil.

In this connection, we obviously had to be interested in the question whether a specific psychrophilic microflora, which would increase numerically during the cold time of the year, might exist in the soil.

Before discussing our original experiments, we would like to emphasize /219 that the existing literature gives no proof of the existence, in nature, of bacterial forms completely adapted to low temperatures. The so-called "psychrophilic" microorganisms described up to now are essentially psychrotolerant forms. For example, most of the molds that are able to grow below freezing, have their optimum temperature near 25 - 30° C. The same is true for bacteria of the genus Pseudomonas, which many authors found to grow at temperatures close to 0° C.

At low temperatures, the growth of such psychrophilic forms is extremely slow. This has recently been indicated by Chistyakov, who studied microorganisms which spoil foodstuffs at low temperatures.

All the above factors make the proposition advanced by Conn somewhat unlikely. In our experiments, we proved that there are no specific microfloras which multiply in soil during the cold seasons.

Using the method of MPA culture, we isolated from Moscow and Crimean soils, in winter and summer at 18 - 20° C, various saprophytic bacteria. The obtained cultures were examined for their temperature ratios, and we found no substantial differences between the "summer" and "winter" forms (Table 59). These experiments demonstrate that the soil contains no bacteria capable of active growth and organic function at low temperatures. The principal soil microflora obviously consists of mesophilic forms, bearing the stamp of the particular climate in which their life cycle takes place.

TABLE 59

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TEMPERATURE BEHAVIOR OF SAPROPHYTIC BACTERIA, ISOLATED FROM SOILS AT VARIOUS TIMES OF THE YEAR

Localities of Soil Sampling	Time of Isolation	Culture No.	Relative Size of Colonies at Temperature (in °C)					
			15	18	25	35	42	46
1	2	3	4	5	6	7	8	9
Moscow . . .	Summer	1	13.2	25.9	83.7	100	0	0
		2	24.4	45.4	81.8	100	0	0
		3	10.4	40.0	82.2	100	0	0
		4	21.1	62.5	94.3	100	75.4	0
		5	37.5	81.8	90.9	100	0	0
		6	5.6	79.6	92.1	100	0	0
		7	9.4	64.6	71.1	100	0	0
Average . .			17.4	51.1	85.1	100	10.8	0.0
Moscow . . .	Winter	1	0	44.1	88.2	100	73.5	0
		2	5.5	41.6	94.4	100	0	0
		3	0	57.1	100	92.8	0	0
		4	23.3	33.3	—	100	0	0
		5	8.3	25.0	94.4	100	0	0
		6	38.4	46.1	100	92.3	0	0
		7	11.4	36.6	73.2	100	0	0
		8	6.2	31.3	37.5	100	0	0
		9	50.0	75.0	77.0	100	70	0
Average . .			15.9	43.3	83.1	98.3	15.9	0.0
South Coast of Crimea . . .	Summer	1	15.3	39.9	66.6	100	0	0
		2	17.6	50.0	66.0	100	50.0	33.3
		3	7.1	57.1	85.7	100	49.0	24.4
		4	11.2	68.1	91.8	100	68.1	0
		5	5.4	28.6	85.7	100	40.0	0
		6	5.8	33.3	80.0	100	77.7	0
		7	4.7	20.0	100	80.0	90.0	40.0
		8	12.3	87.7	100	100	57.1	0
Average .			9.8	48.1	84.5	97.5	53.9	10.9
South Coast of Crimea . . .	Winter	1	0	30.8	62.5	100	83.3	58.3
		2	0	11.4	80.0	100	83.4	0
		3	0	19.2	61.5	22.3	100	0
		4	4.6	43.3	80.0	93.3	100	0
		5	41.7	66.6	100	100	70.8	0
		6	18.7	62.5	100	87.5	93.7	0
		7	8.3	41.6	75.0	100	0.0	0
		8	0	80.0	82.7	100	100	0
Average . .			9.1	44.4	80.2	87.9	78.9	0

It should be mentioned that, in seasonal studies of bacteria, we used a narrow temperature range, which permitted no accurate determination of the position of the optimum. Nevertheless, it is obvious from the above data that bacteria isolated in winter do not show better growth at low temperatures.

We also used the Remi method of microbiological reactions to investigate the capability of the summer and winter microflora of Moscow and Crimean soils of inducing ammonification, denitrification, and decomposition of urea. We are not giving detailed data here, mentioning merely that no noticeable differences exist in the rate of processes initiated by the "winter" and "summer" soil microflora. At low temperatures (10 - 15°) the processes always were very slow. /221

Thus, our experiment demonstrates the dominant role played in soil processes by mesophilic microflora adapted to the local conditions.

In the summer, especially in the South, the temperature rises considerably under the intense insolation. For example, in the Crimea, the Caucasus, and Central Asia, the surface layers of the soil often reach a temperature of 50 - 60° C in the daytime. It is natural to believe that vital functions of the thermophilic microflora, able to live and proliferate in a very high temperature range, would start during such a heat-up period.

At the beginning of our work, we were able to demonstrate the widespread occurrence of thermophilic bacteria in various soils of the USSR. On inoculating several elective nutrient media with soil crumbs and holding them at about 60° C, it is easy to detect thermophilic bacteria, inducing ammonifying, denitrifying, urea-decomposing and other types, in the soils.

We isolated pure cultures of thermophilic ammonifiers from both northern and southern soils, capable of growth in the 35 - 70° C range, with an optimum temperature near 50 - 60° C. We often found thermotolerant forms, with a minimum temperature somewhat lower than that of the thermophiles.

In exactly the same manner, we were able to isolate thermophilic denitrifiers with a minimum temperature near 40° C, an optimum near 55 - 60° C, and a maximum near 70 - 73° C.

The thermophilic forms of denitrifying bacteria have a minimum temperature near 25 - 30° C, an optimum at 45 - 50° C, and a maximum near 60° C.

It was considerably more difficult to isolate thermophilic urea bacteria. Nevertheless, we did succeed in obtaining such forms with a minimum temperature of 30° C, an optimum of 45 - 50° C, and a maximum of 58 - 60° C.

A more detailed study of the role of thermophilic soil bacteria reveals their limited function in soil processes. We based this conclusion on a series of papers. The material at our disposal indicates that the thermophilic microflora had been introduced and was not native to the original soil.

Thus, a survey over the temperature groups of the soil microflora demonstrates that most microbiological processes in the soil involve exclusive parti-

cipitation of mesophilic microflora. This substantiates the existence of an /222 adaptive reaction to the climate by mesophilic bacteria since, at the usual temperature fluctuations of the soil, only the mesophilic group is generally able to function. This also explains why southern bacteria have such a wide temperature range of growth, which would not be necessary at all if a thermophilic microflora existed in the soil.

4. Relation between Principal Temperature Points of Bacteria and Climatic Indices

The collected data clearly point to the regular rise of the growth temperatures of soil bacteria, when progressing from the northerly cold zones to more southerly regions. We were interested in defining the correlation between the climatic indices and the position of the principal temperature points of soil bacteria. We noted above that the individual forms of bacteria are characterized by a certain specificity in their behavior to the growth temperatures. For example, the spore-bearing bacteria of the B.mesentericus group generally have a higher maximum point than asporogenous types. For this reason, in comparing the averages of the growth of various bacteria with the climatic indices, certain deviations in the correlation between these two indices may be noted. Therefore, the law derived above represents merely an index of the general tendency in the adaptive activity of the soil microflora.

TABLE 60

CLIMATIC INDICES AND GROWTH TEMPERATURES OF SOIL BACTERIA

Locality of Soil Sampling	*Optimum Temperature for Bacteria*	*Mean Optimum Temperature*	*Maximum Temperature for Bacteria*	*Mean Air Temperature for July*	*Mean Annual Air Temperature*	*Cumulative Temperature for Hot Period of the Year*
Arkhangel'sk	27—30	28.5	36—38	16	0.6	1600
Leningrad	29—31	30.0	40—42	18	4.1	2050
Moscow	29—31	30.0	40—42	19	4.2	2100
Kursk	33—35	34.0	43—44	20	5.4	2600
Sinel'nikovo	34—37	35.5	43—45	21	9.6	3500
Armavir	35—37	35.5	44—45	21	8.7	3200
Anapa	37—39	38.0	46—47	23	11.7	3400
Balaklava	37—39	38.0	44—45	23	12.1	3600
Massandra	36—39	37.5	44—45	23	12.7	3700
Central Asia (Golodno Step')	37—39	37.0	53—54	27.4	13.7	4500
Batum	38—39	38.5	51—52	24.2	14.7	5300

Table 60 gives certain data on which our comparisons were based. The list is a concise summary of the analytic material of this work and also gives the

mean annual and July temperatures of the various localities.

Table 61 is a comparison of the experimental temperatures of growth of bacteria of various localities, calculated on the basis of:

1) mean annual atmospheric temperature;
2) atmospheric temperature in July;
3) cumulative temperatures for the hot period of the year.

Again, we make the reservation that such a comparison is highly arbitrary for a set of different species, since each microorganism may manifest specific phenomena, characteristic only for itself, in its adaptive activity. We are therefore not disturbed by the occasional deviations in these series. They /223 can be explained by the above considerations as well as by the fact that the climatic elements used in our study cannot possibly reflect its specific nature in full.

TABLE 61

COMPARISON OF EXPERIMENTAL AND CALCULATED OPTIMUM TEMPERATURES OF GROWTH OF SOIL BACTERIA

Locality	Optimum Growth Temperature (in °C)			
	Experimental Value	Calculated from Annual Temperature	Calculated fom July Temperature	Calcul. from Cumul. Temp. for Hot Period of Year
Arkhangel'sk	28.5	-	—	—
Leningrad	30.0	31.1	30.9	30.1
Moscow	30.0	31.1	32.7	30.2
Kursk	34.0	32.1	33.3	32.6
Sinel'nikovo	35.5	35.2	34.6	34.5
Armavir	35.5	34.5	34.6	33.7
Anapa	38.0	36.6	37.0	34.4
Massandra	37.5	37.5	37.0	35.3
Balaklava	37.0	37.0	37.0	35.0
Tashkent	38.0	38.1	42.3	37.7
Batum	38.5	—	—	—

On plotting the relation between the position of the optimum temperatures of bacteria and the above climatic indices in a graph, approximately a straight line will generally be obtained.

The equation of a straight line, according to the mean annual temperature, will read /224

$$\frac{y-28.5}{38.5-28.5}=\frac{x-0.6}{14.7-0.6};\ y=0.74x+28.1.$$

The values calculated on the basis of this equation are in excellent agreement with the experimental results. A similar picture is noted for B.mycoides,

where the only exception was the Igarka race.

The equation derived for the July temperature is

$$\frac{y-28.5}{38.5-28.5}=\frac{x-16.0}{24.2-16.0};\ y=1.22x+8.99.$$

The values calculated from this equation are likewise in good agreement with the experimental data. The exception of the bacteria group from Central Asia is entirely natural. The excessively hot Central Asian summer will of course give too high an index for the calculations.

The following equation is obtained from the cumulative temperature for the hot period of the year:

$$\frac{y-28.5}{38.5-28.5}=\frac{x-1600}{5300-1600};\ y=0.029x+24.2.$$

The values calculated from this equation are slightly too low for several localities but generally agree rather well with the experimental data.

The maximum growth temperatures for the southern cultures are considerably higher than those for the northern. For example, most bacteria from Arkhangel'sk soil stop growing at 36 - 38° C, while bacteria from the soils of the podzol belt have their maximum temperature near 40 - 42° C, those from soils of the chernozem belt near 43 - 50° C, and those from Central-Asian and Batum cultures, near 50 - 53° C.

Plotting the relation between the position of the maximum temperature and the mean annual temperature again yields a straight line with a few (although sharp) deviations for the bacteria from Crimean soil. Of course, this is easy to explain. We only used a relatively small number of bacteria. In a larger collection, such deviations would be obliterated. The fact that the position of the maximum temperature for various species of bacteria of the same climatic zone varies more than the position of the optimum temperatures might also have affected our results.

CHAPTER VIII /225

CLIMATE AND INTRACELLULAR PRESSURE IN BACTERIA

1. Osmotic Pressure in the Bacterial Cell and Analytical Methods

On transition from some climatic zone to another, the moisture supply of the soil usually changes greatly. This must have a marked effect on the osmotic system of soil bacteria, inducing us to prove this proposition by experiment. In individual cases, the influence of the climate may be intensified by specific features of the soils rich in dissolved salts (for instance, in solonchaks).

It should be noted that the literature on the osmotic pressure in the cells of microbes is scanty, especially with respect to the bacterial cell. Nevertheless, it is obvious that this factor plays an exceptionally important role in the vital activity of microbes.

The osmotic pressure of a cell affects not only the uptake of water, but also the state of the plasma and the metabolism (Kholodnyy, Rhode, and others). Any change in the concentration of the cytoplasm will affect the character of the colloidal medium in which the metabolic reactions take place; this may lead to basic modification of the biochemical processes in the vegetal cell.

Factors of this kind considerably complicate the purely physical influence exerted by the osmotic properties of the medium on the cell.

Some bacteria are able to grow on distilled water, while others will grow on highly concentrated salt solutions.

According to Czapek, the osmotic pressure in the bacterial cell is usually 3 - 6 atm, and rarely exceeds 20 atm. When grown on concentrated solutions, the osmotic pressure in the cell of the microorganism may be considerably higher, going as high as 300 atm or even beyond in individual cases.

Lewandowsky (1904) succeeded in isolating, from garden soil, bacteria /226 that developed in a 23% NaCl solution or in a saturated solution of saltpeter. Laurent noted long ago that certain yeasts are able to grow in a 60% sugar solution. Several investigators (Wehmer, Cleyfert, Muller-Thurgau et al.) have stated that the maximum osmotic pressure of the medium at which microbes are able to grow is in the range of 60 - 95 atm. This figure obviously is too low. Some authors have noted not only that individual microbes show differing behavior toward an increase in concentration of the solution, but even that individual cells of the same culture may react differently, depending on their age and on the medium (see the summary by Pantanelli, Laurent, Eschenhagen).

Observations by Kostychev and Kholkin on soils of Central Asia show that certain soil processes, such as ammonification, do not yet stop at an osmotic pressure of the solution as high as 80 atm or higher. However, nitrifying bac-

teria no longer function under these conditions. Hence, it can be concluded that by no means all bacteria behave in the same way toward an increase in concentration of the soil solution.

According to Eschenhagen (1889), Aspergillus niger, Penicillium glaucum, and Botritis cinerea may be grown on a 51 - 55% glucose solution, on a 37 - 43% glycerin solution, and on a 12 - 18% NaCl solution.

In comparatively recent work, Walter (1927) using an original method of study, found that the following osmotic pressure can develop in the cells of individual microbes:

Aspergillus glaucus	up to 220 atm;
Penicillium glaucum	up to 220 atm;
Rhizopus	up to 90 atm;
Bacteria	up to 50 atm.

These figures are very close to the data given by Waldendorff.

It may be concluded from the above data that the osmotic pressure in the cell of microbes may be extremely great. For this reason, the microorganisms comprise a considerable number of forms capable of growing on rather concentrated media. Fungi, in general, are more osmophilic than bacteria. Table 62 gives the concentrations of mineral salts tolerated by lower organisms (according to Lyubimenko).

TABLE 62

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EXTREME CONCENTRATIONS OF SALT TOLERATED BY VARIOUS MICROORGANISMS

Type of Microorganism	*Concentration of Salt Stopping Growth of Microorganism (in %)*	
	NaCl	$NaNO_3$
Saccharomyces Zopphii	12	—
Penicillium glaucum	17—20	21
Aspergillus niger	17	21
Aspergillus glaucus	26	46
Mucor racemosus	—	20 KNO_3
Chlamydomonas Ehrenbergii	5	21 $MgSO_4$
Tetraspora explanatum	10	—
Pichia membranafaciens	—	80

According to data by Eschenhagen, molds tolerate the extreme concentrations of various substances as given in Table 63.

A number of references to halotolerant microorganisms may be found in

papers by Fitting, Euler, Palm, Fritsch, Matzuschita, Baranik-Pikowsky, Kraemer and Krumbholz, Sperlich, Stewens, Lumstein, Petrova, Golikova, Rubenchik, and Gal'perin.

TABLE 63

EXTREME CONCENTRATIONS OF CERTAIN SUBSTANCES STILL ALLOWING GROWTH OF MOLDS (IN %)

Type of Mold	$C_6H_{12}O_6$	$C_3H_6O_3$	$NaNO_3$	$CaCl_2$	NaCl
Aspergillus niger	56	43	21	18	17
Penicillium glaucum	55	43	21	17	18
Botritis cinerea	51	37	37	16	12

When microorganisms are cultured on strong salines, they become adapted to the osmotic pressure of the medium as well as to a relatively high concentration of salt. This is due to the fact that a strong salt solution increases the osmotic pressure and promotes an accumulation of excess salt in the cell. It is entirely conceivable that, occasionally, the second of these causes, rather than the first, might retard the growth of a given microbe.

This is distinctly reflected in the experiments by Voytkevich (1928) on /228 Penicillium glaucum and Oidium lactis. He showed that Penicillium glaucum, on which NaCl does not have a specific effect, will stop growing at an NaCl concentration of about 28 - 30%.

The same organism stops growing at 80% relative atmospheric humidity (corresponding to 30% NaCl), in experiments made in a Böttcher moist chamber. Thus, these two methods give results entirely in agreement.

However, the other microorganism, Oidium lactis, which does not tolerate high NaCl concentrations, stops growing at 12.3% NaCl in the medium. In Böttcher's chamber, it suspends growth at 83 - 84% relative humidity, corresponding in osmotic pressure to 25 - 26% NaCl. The discrepancy between the results of the two methods in this case may be explained by the fact that Oidium lactis does not tolerate large amounts of NaCl but that, at the same time, it is able to grow on a physiologically sufficiently dry medium.

Sugar, organic acid, and mineral salts have an osmotic effect in the vegetal cell. According to the relative content of these substances in a given cell, a rough estimate can be made as to the differences in the osmotic pressure in microorganisms. The following are data on the variations of the ash percentage in microbes, calculated from the dry weight of their cells:

	Minimum	Maximum
Bacteria	1.77	31.0
Yeast	2.0	7.0
Higher fungi	4.3	15.0

The ash content, for understandable reasons, usually is higher in halophiles and marine plants.

Findings were reported that the lower organisms quite readily and rapidly adapt to varying concentration of the external medium. In this connection, it should be noted that the range from minimum to maximum concentrations tolerated by the organism most likely narrows in passing from lower forms to higher.

Despite the ready environmental adaptation of microbes to the medium, it does not seem that their acclimation, especially in individual forms, proceeds with excessive speed.

Thus, A.Fischer (1895) noted that, on transfer of B.anthracis, B.coli, and B.cholerae from a solution containing 0.73% NaCl to a solution with 2% NaCl, these bacteria die within 10 - 60 min. On gradual adaptation, however, they /229 can live in solutions containing 5 - 7% NaCl. Luminous bacteria may cease to emit light on transfer to a salt solution weaker than that of the sea (Johnson and Newton). In other forms of microorganisms, a rapid rise or drop in the concentration of the medium causes a more or less prolonged cessation of growth, after which development is resumed.

The interesting question as to the regulation of osmotic pressure in the cells of microorganisms has not been sufficiently elucidated.

As to the mechanism of the adaptivity of the cell to the osmotic pressure of the external medium, the opinions of various authors differ considerably. Some believe that the regulation of the intracellular pressure takes place by means of a diffusion process (Fischer, Drews, Swellengrebel, Artari, and others). Others believe that the regulation takes place by diffusion as well as by formation of substances that increase the cell turgor. The latter substances are usually believed to play the principal role (Pantanelli, Eschenhagen, Alekseyev, and others).

Finally, some authors consider that regulation in solutions of a nonelectrolyte is accomplished by the formation of turgorogens, and in a solution of an electrolyte by diffusion (Buchheim, Euler).

It is difficult to say how rapidly the equalization of the concentration of an external solution proceeds, on its conversion to the cell contents of a given microbe, since the views differ in this respect.

Fischer, in his work on bacteria, emphasized the rather rapid deplasmolyzation of their cells in solutions of various substances, which would point to a high permeability of the bacterial cell.

The duration of plasmolysis in the Fischer experiments was often measured

in minutes. For example, on placing Spirillum undula and Cladothrix dichotoma into plasmolyzing solutions, he regularly observed a rapid onset of the reverse process (deplasmolysis) in the same solution. According to his data, deplasmolysis in a 1.25% NaCl solution in individual bacterial cells already sets in after 3 - 4 min, but more generally the distension of the protoplast took place in 15 - 30 min, although single plasmolyte cells could still be observed two hours after the beginning of the experiment. In a 5% KNO_3 solution, plasmolysis disappeared within 5 - 7 min in most of the cells, and in a 7.5 - 10% KNO_3 solution, deplasmolysis set in so rapidly that, within 3 - 4 min after being placed into the solution, most cells of Spirillum undula and Cladothrix dichotoma had already returned to being homogeneous. In this connection, Fischer writes: "For the protoplast again to be able to expand, the internal pressure in the /230 cell must be higher than the pressure of the surrounding solution. This can occur, either if the solution penetrates readily through the cell wall and the plasma membrane, or if the properties of the protoplasm are such that a substance with a high osmotic power is developed in the cell. If the latter occurred, then plasmolysis would persist for some time."

A similar statement was made by Euler and Palm, who worked on the plasmolysis of yeasts. "We are inclined to believe" they write, "that, from our experiments and from the earlier literature, the adaptation of yeasts to unusual osmotic pressures consists primarily in a change in the permeability of their plasma membrane. Because of the penetration of substances from the highly concentrated solutions surrounding the yeast cells, to which the yeasts adapt themselves, this difference in osmotic pressure within and without the cytoplasm is leveled out."

It should be noted that Swellengrebel (1905), in studying the permeability of the yeast cell, was unable to confirm Fischer's conclusion of high permeability of the cell to substances plasmolyzing it. Only certain compounds, such as urea and dextrose, gave a picture of rapid deplasmolysis in yeasts, while many other mineral and organic compounds behaved in an entirely different way. It therefore seems that Fischer's conclusions require further substantiation.

Interesting data on the regulation of osmotic pressure in microorganisms can be found in Eschenhagen's paper on the Aspergillus mold. When this mold was transferred from a solution with 1% saccharose to a solution with 34% of the same sugar, a marked increase in osmotic pressure in the cells of the mycelium was observed within one hour. It was also established that the ratio

$$\frac{\text{concentration of salt inducing plasmolysis}}{\text{concentration of substances in medium}}$$

when the organism was grown in various media was rather constant, ranging from 1.5 to 1.9. Pantanelli, however, found that this ratio increased for Aspergillus niger to 3.06 - 3.93.

Seliber and Katznelson found that an increase in the concentration of the medium increased the osmotic pressure of the yeast cell. This increase, however, was not always proportional to the concentration of the nutrient medium.

Usually, an increase in the osmotic pressure of the medium causes a marked

change in the biochemical activity of microorganisms. The purely chemical /231
action of the substances on the cytoplasm is cumulative with the increase in the osmotic pressure itself.

Only a few authors have studied the effect of the osmotic pressure of the medium on the activity of microorganisms. We mention the work by Brown, by Rubner, and by Kublanovskiy, who studied the relation between the enzymatic action of yeasts and the composition of the medium.

Richter investigated the effect of various equimolecular solutions on the osmophilic yeast Zygosaccharomyces mellis acidi described by him. Kollegorskaya did similar work on Saccharomyces cerevisiae and Zygosaccharomyces mellis acidi.

Johnson and Newton studied the influence of the osmotic pressure of the medium on the vital activity of photobacteria. Their luminescence diminished more than their respiration in diluted seawater. In more concentrated solutions the reverse picture was noted. In very weak and very strong solutions of seawater, luminescence, respiration, and viability of photobacteria were all rapidly suppressed, but this suppression was reversible.

One must finally mention the work done by Artari on the growth of the halophilic algae Asieromonas gracilis and Dulaniela salina in salt solutions.

A considerable number of authors have considered the extent of osmotic pressure in the lower and higher organisms to be an ecological trait, resulting from environmental conditions and transmitted to the progeny (Blagoveshchenskiy, Dumeche, Boldyreva and Grinbaum, Dianova and Voroshilova, Maksimov and Lominadze, Delyanin and Silikova, and others).

Some investigators have emphasized, however, that although the osmotic pressure does vary with the environmental conditions, its magnitude is nevertheless a systematic trait rather than an ecological one (Seliber, Katznelson, Lambrecht).

Kublanovskaya noted that the optimum osmotic pressure of a medium may be different even for genetically similar organisms. For example, the optimum saccharose concentration for Saccharomyces cerevisiae (Race XII) was 5%; for Saccharomyces Frohberg and Saccharomyces ellipsoideus Steinberg, it was isotonic with a 15% saccharose solution.

With respect to the life of plant organisms in the soil one arrives a priori at the conclusion that specific features of the location of a given soil must leave an impression on them. For example, Il'in and coworkers found /232
that the osmotic pressure in plants of moist localities was lower than in plants of dry places. It is, of course, difficult to assume that climatic and soil conditions would not, in some way, affect the osmotic pressure in the cells of microorganisms.

In the present study, we analyzed the magnitude of the osmotic pressure in certain soil bacteria. Main emphasis was placed on B.mycoides, as the bacteria selected previously as our preferred object for other tests.

2. Concept of Intracellular Pressure

Unsprung and Blum (1920) stated that, as a rule, the definition of osmotic pressure of the plant cell, simple though it might seem, is misunderstood. Usually, if a solution of saccharose or NaCl induces marginal plasmolysis, it is said that the osmotic pressure of the cell is isotonic to that solution. Again, osmotic value, or some such term, is often used as a synonym for osmotic pressure.

Yet it is obvious that the actual osmotic pressure of a cell is determined not only by the intracellular substances with an osmotic action, but also by the concentration of the external solution. Let us explain this on an example. Let a cytoplasm have a concentration isotonic to 0.5 mole of sugar. Exactly such a solution is contained in the medium surrounding the cell. In determining the osmotic pressure by the conventional method, plasmolysis will be encountered at a sugar concentration somewhat higher than 0.5 mole, so that it would seem that considerable osmotic pressure prevails in the cell.

However, if the external and internal solutions are isotonic, then the osmotic pressure in the cell will be zero. It is true that the cell has the potential power to manifest a certain pressure, but this could become fully manifest only in a solution of pure water.

This makes it clear that a determination of the osmotic pressure of a given cell is possible only if the concentration of the osmotically active substances, both inside the cell and in the solution surrounding it, is taken into consideration.

Starting from similar arguments, Unsprung and Blum proposed the following terminology for describing osmotic phenomena in the plant cell.

1) Osmotic value, indicating the concentration of a solution (saccharose, NaCl, etc.) isotonic to the cell contents. This quantity can be expressed /233 in moles or in atmospheres.

The osmotic value can be determined on the basis of: a) marginal plasmolysis; b) decrease in cell volume in a salt solution. These two methods give somewhat differing values, but objective reasons often require the application of one or the other of these methods. In particular, in bacteria and yeasts, marginal plasmolysis often does not occur at all or is observed only in solutions known to be hypertonic. Therefore, we believe that the osmotic value in the latter case is best determined from the decrease in cell volume.

2) Osmotic pressure, i.e., the pressure manifested by the osmotically active cell contents on the cell wall, expressed in atmospheres. The osmotic pressure results from the osmotic value of the plant cell and the concentration of the surrounding medium. It is entirely obvious that the same cell, at unchanged composition, may change its osmotic pressure when transferred from a medium with one concentration to another medium richer or poorer in osmotically active compounds.

3) Turgor pressure, a quantity expressed in atmospheres and showing the

total pressure of the cell contents against the cell wall. In plant cells, the absolute values of the osmotic and turgor pressures are often almost the same.

4) Membrane pressure, a quantity opposing the turgor pressure and manifested by the cell wall.

5) Suctorial force of the cell, a quantity expressed in atmospheres and representing the force with which the cell can take in water from a certain surrounding medium.

From these definitions, we can omit some, as irrelevant to the present work.

Some of these factors are interrelated in a definite manner. For example, we see from the equation that:

S	=	O	-	T
Suctorial force		Osmotic value		Turgor pressure

Hence it is clear that when the cell has zero turgor pressure (for example when it is plasmolyzed), then the absolute value of the suctorial force will equal the osmotic value; in other words, the cell will have a great potential suctorial force. /234

When the cell is immersed in water, its suctorial force is zero, and the osmotic value corresponds to the turgor pressure.

The term "osmotic value" cannot be considered particularly apt for bacteria, since we have no reliable knowledge as to the relative role played by the cell sap and the cytoplasm in the retention of water by the bacterial cell. The concept of "osmotic value" assumes that the dominant role is played by the cell sap. Yet the work of Walter has established that the change in cell volume is often explained by the swelling of the plasma. When bacteria are the object of study, we cannot delimit the role that the phenomena of osmosis and swelling play in the retention and loss of moisture, and must assume that both phenomena have a definite significance here. We therefore would suggest to replace the term "osmotic value" by "intracellular pressure". This term would include the total force of both the energy of swelling and that of the osmotically active substances of the cell. If the cell has large vacuoles and the role of the cytoplasm is negligible, "intracellular pressure" would be practically equal to "osmotic pressure". In the opposite case, the phenomenon of swelling would have to be assigned the primary role.

In working with bacteria, it is impossible to separate these phenomena (osmosis and swelling) so that, instead, the pressure caused by both factors on the cell wall is determined.

3. Applicability of Existing Methods for Osmotic Pressure Determination, to Bacteria

In establishing the magnitude of the osmotic intracellular pressure in the microbial cell, the principal difficulties are of a methodological nature. We will not be wrong in stating that there is no perfect method today for studying bacteria according to this property. The subject is somewhat better developed for the yeasts.

Of the existing methods, applied to investigations on higher plants, not one is entirely suitable for bacteria, nor, in all probability, for yeasts. The drawbacks of these methods, from the viewpoint of bacterial studies, will be pointed out below in our discussion on the methods of greatest interest for our work.

Plasmolytic method. Of the various methods for determining the osmotic pressure, the plasmolytic pressure of de Vries is generally known. This method is in wide use for measuring the osmotic pressure of plant cells. /235

Without further describing this well-known method, we note merely that, even when applied to higher plants, it sometimes leads to serious errors. Its principal shortcoming results from the fact that the cell wall, in a state of turgor, also resists the osmotic pressure by virtue of its elastic properties. Therefore, if a cell is placed in a weakly hypertonic solution, its volume will decrease, but it is always possible that this decrease will not involve detachment of the cytoplasmic layer, since the cell wall will also contract. Only in stronger solutions, where the elasticity of the cell wall decreases on marked contraction of the protoplast, will it be possible to observe the phenomenon of plasmolysis. For example, according to Krasnosel'skaya-Maksimova, in the sunflower, 30% of the water of the cell sap can be removed without marked change in the state of turgor.

The error due to this cause will be greater in young cells with a more elastic cell wall than in aged plant cells.

Judging from a number of papers mentioned below, the phenomenon of plasmolysis can also be observed in microbes (bacteria and yeasts), but its course here will be somewhat different from that in the cells of higher plants. For example, in one of the earliest papers by Fischer on this question (1891-1895), the possibility of inducing plasmolysis in bacteria is demonstrated. At that time, this proposition was of fundamental importance, since it served as a justification for criticizing the views expressed by Büchli on the structure of the bacterial cell. The latter author admitted the presence of only a nucleus and wall in bacteria.

Fischer pointed out that plasmolysis in many bacteria can often be observed when a preparation is dried on a coverglass, increasing the concentration of the surrounding solution (preparation plasmolysis). Plasmolysis in preparations is easily observed at low salt concentrations in Spirillum undula, B.fluorescens, B.Solmsii, and other organisms. In plasmolysis, the protoplast contracts and concentrates at the cell poles, forming the so-called polar granules. In

general, according to Fischer, the long rods, vibrions, and Spirillae are easily plasmolyzed; round bacteria and short rods give a very obscure plasmolysis.

It is of interest that in certain bacteria, such as B.subtilis, B.megatherium, B.anthracis, etc., Fischer did not succeed in inducing plasmolysis. Here he noted only certain cytoplasmic changes, which showed in increased refractivity.

On the basis of his work, Fischer divides the bacteria into two groups. /236 The first group, namely, the plasmolyzing type, has a protoplasm impermeable to a number of plasmolysis-inducing substances. The protoplasm of the second group of bacteria is highly permeable to many substances so that, when submerged in these materials, the cells do not show plasmolysis.

As we will show below, this proposition cannot be regarded as a satisfactory explanation for the absence of plasmolysis in certain bacteria.

Fischer noted that, in many bacteria, deplasmolysis sets in rather fast. Thus, in Spirillum undula in 2% KNO_3, strong plasmolysis is noted in half a minute, but within four minutes the cell begins to deplasmolyze in the same solution, and strong deplasmolysis sets in after 20 min. Deplasmolysis is less marked in the cells of Cladothrix. While 10 hours after start of plasmolysis all cells of Spirillum undula had been completely deplasmolyzed in KNO_3, many cells of Cladothrix dichotoma were still plasmolyzed after this time.

The rate of deplasmolysis depends on the composition of the solution. In an NaCl solution it was higher than in KNO_3, but was still lower in a solution of cane sugar, which in all probability had to do with the rate of penetration of substances into the cell. It is also noted that, in stronger salt solutions, deplasmolysis is generally faster than in weak solutions. Washed bacterial cells (after deplasmolysis) are again able to manifest plasmolysis. This definitely shows that the regulation of osmotic pressure in the microbial cell takes place not by the production of osmotically active substances, but by the passage of certain quantities of salts into the cell.

It seems that the Fischer experiments on the rate of deplasmolysis should have been checked, since results contrary to his conclusions were obtained on yeasts.

Besides this work of Fischer on the plasmolysis in bacteria, others should be mentioned. Thus, Migula observed plasmolysis in the large B.oxalaticus in which the protoplasm detached completely from the cell wall and the central vacuole decreased to an invisible size.

Plasmolysis has also been observed by other authors in various preparations. For example, Toni and Trevesin mentioned the appearance of plasmolysis in representatives of the genus Pasteurella. Flügge noted the same phenomenon in the bacteria of dental deposits. Rahmer described the so-called tinction phenomenon in cholera vibrio which, in Fischer's opinion, is nothing else but /237 plasmolysis. Plasmolysis is readily noted in many bacteria listed in the Fränkel-Pfeiffer Atlas (the etiologic agents of tuberculosis, cholera, fowl

cholera, etc.).

It should be noted that, in the staining of plasmolyzed bacteria, signs of plasmolysis very often disappear, most likely due to the fact that the dye causes swelling of the protoplast.

Large Spirillum cells have often been used as study objects. Plasmolysis in Spirillum giganteum has been observed by Ellis and Swellengrebel. Vahle noted plasmolysis in Spirillum volutans, and Reichel in Spirillum undula. These authors noted in the Spirillum cell a shrinkage of the protoplast and its division into separate isolated parts. In many bacteria, the typical picture of plasmolysis cannot be observed. Gabrowsky, when placing cells of Vibrio proteus in 1% and 5% NaCl solutions, was unable to note any detachment of the cell contents from the cell wall.

Hinze reported contraction of cells of Beggiatoa mirabilis and Thiophysa volutans in hypertonic solutions, without appreciable detachment from the cell wall. Ruhland and Hoffmann likewise confirmed Hinze's results on Beggiatoa mirabilis. Hölling reached the conclusion, based on his own experiments, that spirilla, in contrast to spirochaetae, are plasmolyzed.

Vahle showed that cells of mycobacteria, in concentrated solutions of electrolytes, do not show the picture of plasmolysis. The change taking place here is of the type observed in cells without a membrane.

Recently, Imshenetskiy published a paper on plasmolysis in sporogenous bacteria (B.mycoides, B.megatherium, B.tumescens, and B.subtilis). These forms, in Fischer's opinion, should not show plasmolysis.

Imshenetskiy proved that Fischer's conclusion was not entirely correct. According to Imshenetskiy, sporogenous bacteria in hypertonic solutions undergo morphological changes, proceeding in two phases. The first phase, which occurs in NaCl solutions of concentrations not above one mole, is characterized only by a decrease in cell volume. When the concentration of the solution is increased (to 4 moles), a further decrease in the volume of the bacteria is noted; in some of the cells, at the same time, the cytoplasm separates from the cell wall (plasmolysis), corresponding to the second phase of the changes.

These facts permit the conclusion that the cell wall of sporogenous bacteria possesses considerable elasticity. A decrease in the cell volume of /238 such bacteria by 50 - 60% may fail to be accompanied by detachment of the protoplast from the wall. A similar conclusion was drawn by us before publication of the above paper (1937).

Imshenetskiy states that the changes in plasmolysis are reversible and have little effect on the state of the cell. The cells of killed bacteria in hypertonic salt solutions do not decrease in volume and do not undergo plasmolysis.

We made experiments on Azotobacter chroococcum. This large bacterium in hypertonic solutions (although detected only with difficulty) shows shrinkage of the entire cell but without appreciable plasmolysis. For the smaller bacteria, which constitute the overwhelming majority, we do not believe the plasmo-

lytic method suitable for osmotic pressure determinations. This becomes more evident if we recall the extreme elasticity of the bacterial cell wall.

Thus, to summarize the above observations on bacteria plasmolysis, we may conclude that this phenomenon proceeds in an entirely different way in bacteria than in the higher plants. The typical picture of plasmolysis is observed in very few bacteria and, to judge from the work of Fischer, proceeds rather rapidly. In some bacteria and perhaps in many, no plasmolysis is observed at all or only in extremely strong salt solutions (Imshenetskiy). It was impossible to study plasmolysis in more detail on small bacterial forms, since the available data cannot be accepted as adequate.

Walter, in discussing the phenomenon of plasmolysis, noted that the following conditions are necessary for plasmolysis to occur:

1. The cytoplasmic membrane must be more or less impermeable to the substances in solution.

2. The cell wall must be sufficiently strong and must not contract to follow the shrinking protoplast.

3. The cytoplasm must be deformed readily and must not have too great a volume by comparison with the cell sap, since if this is the case the cytoplasm might swell and cancel the plasmolysis.

Hence, it is easy to conclude that, for example, the elasticity of the cell wall in bacteria and yeasts makes the plasmolytic method of determining the osmotic pressure unsatisfactory.

In particular, not only in bacteria but also in algae, especially in soil algae, no plasmolysis occurs, again for the above reasons. In some algae, however, other factors also inhibit plasmolysis, such as, for example, the strong adhesion of the thin cell wall to the protoplast, the presence of a swelled /239 membrane (Bangia), etc.

A study of several papers on plasmolysis in yeast cells showed that it is extremely difficult to find guidelines for establishing the magnitude of osmotic pressure in such cells. Interesting data on this question are contained in the books by Euler and Palm. These authors noted that young yeast cells in general are less readily plasmolyzed than old cells. It is interesting that rather concentrated solutions of glycerin contain both distinctly plasmolyzed cells and unchanged ones. Thus, in 10% glycerin, 17.1% of the cells were plasmolyzed; in 15% glycerin, 25.7%; in 20% glycerin, 38.4%; and in 25% glycerin, 71%.

It is obviously difficult, on the basis of the data obtained, to determine the concentration of glycerin that would be isotonic to the cell sap of yeasts.

Swellengrebel states that it is not easy to detect plasmolysis in yeasts because of the fact that the cell membrane is highly elastic and contracts together with the protoplast. Only in more concentrated solutions can a clear-cut picture of plasmolysis be noted. Even if there is such a picture, the cytoplasm does not completely separate from the cell wall and remains connected to

it by strands of cytoplasm.

To obtain data closer to the truth, he recommends that, in defining the isotonic concentration, the investigator should not rely on signs of definite plasmolysis but on the first appearance of separation of cytoplasm between mother and daughter cells in budding individuals. From his own experiments, Swellengrebel found that, if the yeast culture is young (24 hours) and if certain salt concentrations are used, not all cells will give the picture of plasmolysis. He also found that the composition of the nutrient medium on which the yeasts were grown affected plasmolysis, by changing the concentration of the isotonic salt solution.

His observations on the plasmolysis in yeasts are interesting. He found, after testing a considerable number of compounds, that plasmolysis set in within 24 hours after beginning of the experiments, but only in solutions of dextrose and urea. In solutions of sodium chloride, nitric acid, and other inorganic compounds, he encountered no plasmolysis.

In our opinion, the irregular plasmolysis of individual yeast cells observed by the above authors can be explained by their capability of strong contraction (cf. infra), whereas the separation of the protoplasm from the cell wall will accompany such shrinkage only in more or less aged cells.

In our experiments, a young culture of beer yeast was placed on an object glass in a strongly hypertonic solution (about 2 N) of sodium chloride and /240 sugar. After a short time, an extremely strong shrinkage of the cell set in, but plasmolytic phenomena were observed only in isolated individuals.

In an old yeast culture, under the same conditions, most of the cells showed plasmolysis, but accompanied by a strong decrease in volume. These experiments, described later, showed that is is more reliable to determine the osmotic pressure in yeasts from the changes in volume; this method, which Seliber selected for his work, seems entirely suitable (cf. infra).

This fact makes plasmolysis an unreliable indicator for determining the osmotic pressure in yeasts and bacteria. In bacteria, aside from other considerations, plasmolysis is very difficult to detect. This makes it logical that many investigators tried different methods for determining the osmotic pressure in the microbial cell.

Höfler method. For cells whose protoplast either difficultly detaches from the membrane or is insensitive to a slight hypertonia of the external solution, Höfler proposed an original method of investigation. He recommended the use of strongly hypertonic solutions for plasmolysis and measuring the volume of the resultant protoplasmatic sac. Obviously, the volume of the cell must also be measured before plasmolysis.

On the basis of his data, Höfler came to the conclusion that the decrease in volume of the protoplasmatic sac in plasmolysis is proportional to the increase in concentration. If this is so, then, if the concentration (c) of the solution used for plasmolysis and the volume of the cell in the hypertonic solu-

tion relative to the original volume (b) are known, the concentration of the solution isotonic to the cell sap can be calculated. This will be equal to the product c·b.

Seliber and Katznelson, based on Höfler's data, believed that the osmotic pressure in the yeast cell equals the concentration of the salt solution causing plasmolysis, multiplied by the ratio of the cell weight in the state of plasmolysis to the weight of a normal cell or, which comes to the same, by the ratio of the number of cells per unit weight of normal yeast to the number of cells per unit weight of plasmolyzed yeast.

As an example, if 10 billion cells per gram are counted in a culture and if, after immersion in 6% NaCl, 12 billion cells are counted for the same weight, then the osmotic pressure of the yeast cell in the culture will be /241
6 × 10:12 = 5.0, i.e., 5% NaCl.

The Höfler method apparently cannot be applied to the study of bacteria, because of the small size of the bacterial cells.

It should be noted that, in checking the Höfler method for yeasts, Seliber and Katznelson found several deviations from his rule. Thus, it was to be expected that with increasing concentration of the solution the number of cells per unit weight would gradually increase, since their volume would decrease. Instead, the authors observed a certain periodicity whose nature they could not explain.

A number of observations indicate the existence of rather frequent deviations from the Höfler rule in work on more highly organized organisms. This was noted by Walter in investigating the marine alga Bangia.

Euler and Palm emphasized the possibility, in working with the Höfler method, that not only the cell sap will lose water, but also the protoplasm.

Hedin method. We must also mention the Hedin method, developed for animal tissues without solid membranes, where it is convenient to determine the water intake or outgo from the change in volume. For example, to measure the pressure of red blood cells, Hedin recommends centrifuging the same quantities of blood in the hematocrit, with salt solutions of different concentrations. The cell contents of the erythrocytes will be isotonic with the solution that does not change their original volume.

Although the Hedin method is recommended for cells without membrane, it seems that methods similar in principle to Hedin's method should be most acceptable and technically easy for work on bacteria and yeasts. We will justify this proposition in more detail in the next Section.

Naturally, even this method is not without shortcomings which, however, are also inherent to the plasmolytic method.

Presumably, water is lost during shrinkage and plasmolysis of a cell not only by the cell sap but also in part by the protoplasm, part of whose water is

readily given up. For example, Walter proved this hypothesis for the alga Lemanea, which has no vacuoles but shows shrinkage of the cell in salt solutions. Obviously, this proceeds here exclusively because of the loss of water from the cytoplasm.

Gravimetric method of Seliber and Katznelson. In one of their papers /242 on the determination of osmotic pressure in the yeast cell, Seliber and Katznelson proposed a gravimetric method, which essentially is a modification of the Hedin method. They proceeded as follows: One gram of pressed yeast was weighed out into weighed tubes. Then, NaCl solution of varying concentration was poured into the various tubes. After 30 min, the tubes were centrifuged for 15 min, the liquid was poured out, and the tubes were cleaned of water with filter paper, taking care to avoid contact between filter paper and yeast.

The tubes were then weighed again to find when the yeast swelled and when it lost water.

The Kalyuzhniy method. Kalyuzhniy, an associate of Seliber, in studying the possibilities for determining the osmotic pressure in the yeast cell, came to the conclusion that determination of osmotic pressure by the plasmolytic-volumetric method of Höfler or by the gravimetric method of Seliber and Katznelson, were both based on incorrect assumptions, since they had taken into consideration the volume and weight of the whole cell, instead of the volume and weight of its osmotically mobile part. This led to a certain error in the corresponding conversions.

Kalyuzhniy treated pressed yeast with various solutions of NaCl and with the crystalline NaCl. The water removed from the yeast was measured in a specially designed and rather simple apparatus. Volumetric measurement of the solution is quite accurate and permits defining the difference in the osmotic action on the cell not only by different concentrations but also by different volumes of the same solution.

Volumetric measurement of solution, before and after plasmolysis, and use of the formula

$$a = \frac{m \cdot 100}{V + \Delta V}$$

permits determining the new concentration of the surrounding solution which is isotonic to the concentration of the cellular sap. In this formula, a is the index of isotonic concentration, m the plasmolytic mass in grams, V the initial volume of the solution, and ΔU the increment of volume after plasmolysis.

Kalyuzhniy found that the removal of water from the cells by solution-induced plasmolysis is dependent on the concentration of the cell sap, and /243 also on the number of cells per unit weight.

The volume of water removed from the pressed yeast as a result of the action of crystalline NaCl form increases to a maximum, after which, regardless of the increase in weight of the salt, it remains constant. The maximum quantity of water removed by crystalline NaCl from fresh pressed yeast, in the pre-

sence of 70% water, was 53% of its weight.

Thus, all the water of the cell is divided by plasmolysis into osmotically mobile and osmotically fixed water, the osmotically mobile water in pressed yeast being about 50 - 53% and the osmotically fixed, about 22 - 24%.

Based on the established content of osmotically mobile and osmotically fixed water in the cells, a new method of determining the osmotic pressure of the cell sap of yeast has been developed.

The osmotic pressure is calculated from the formula

$$A = \frac{m \cdot 100 (b - \Delta b)}{(V + \Delta V) b},$$

where b is the initial volume of water in cc, in the weighed portion of yeast, $\Delta b - V$ is the volume in cc of water removed from the weighed portion of yeast; this means that the initial concentration of the cell sap equals the index of isotonic concentration, multiplied by the ratio of the remaining osmotically mobile water in the cell to its initial content.

Thus, to determine the osmotic pressure in the yeast cell, we must directly determine the osmotically mobile part of the cell.

Seliber mentioned that, if we have the data on the loss of weight of the cell in plasmolysis in two concentrations of plasmolyzing solution, it is possible, without direct determination, to define the osmotically mobile part of the weighed portion of yeast (or of a yeast cell) and then the actual magnitude of the osmotic pressure in the cell.

Using the following notation:

P = weighed portion of yeast,
P_1 = weight of this portion after treatment with a solution of concentration C,
P_2 = weight of this portion after treatment with a solution of concentration C_1,
O = osmotic value of the yeast cell,
K = portion of the cell of constant weight (remaining after removal of the osmotically mobile portion), then these data can be used for constructing the following equation: /244

$$O = \frac{c(P_1 - K)}{P - K} = \frac{c_1(P_2 - K)}{P - K}; \quad c(P_1 - K) = c_1(P_2 - K);$$

$$cP_1 - cK = c_1P_2 - c_1K; \; c_1K - cK = c_1P_2 - cP_1; \; K = \frac{c_1P_2 - cP_1}{c_1 - c}.$$

Knowing K, the osmotically mobile part of the cell can be determined, and the value of O can be obtained from the formula

$$O = \frac{c(P_1 - K)}{P - K}.$$

Substituting the value of K into this equation, we obtain

$$O = \frac{c\left[P_1 - \frac{(c_1P_2 - cP_1)}{c_1 - c}\right]}{P - \frac{c_1P_2 - cP_1}{c_1 - c}} = \frac{c(c_1P_1 - P_1c - c_1P_2 + P_1c)}{c_1P - cP - c_1P_2 + cP_1} =$$

$$= \frac{cc_1(P_1 - P_2)}{c_1P - cP - c_1P_2 + cP_1}.$$

In determining the osmotic pressure from the weight change of the yeast cell, when the number of cells in 1 gm of plasmolyzed and unplasmolyzed yeast is actually used for the calculation, the value of O can be found by substituting, in the above formulas, the quantities P, P_1, and P_2 by the weight of the yeast cell, i.e., 1 gm divided by the number of cells in one gram of plasmolyzed and unplasmolyzed yeast.

Using the following notation:

n = number of cells, in billions, in 1 gm of unplasmolyzed yeast,
n_1 = number of cells, in billions, in 1 gm of yeast plasmolyzed in a solution of concentration C = c,
n_2 = number of cells, in billions, in 1 gm of yeast plasmolyzed in a solution of concentration $C_1 = c_1$, the following values are obtained for K and O:

$$K = \frac{c_1P_2 - cP_1}{c_1 - c} = \frac{\frac{c_1}{n_2} - \frac{c}{n_1}}{c_1 - c} = \frac{c_1n_1 - cn_2}{n_1n_2(c_1 - c)}.$$

$$O = \frac{cc_1(P_1 - P_2)}{c_1P - cP - c_1P_2 + cP_1} = \frac{cc_1\left(\frac{1}{n_1} - \frac{1}{n_2}\right)}{\frac{c_1}{n} - \frac{c}{n} - \frac{c_1}{n_2} + \frac{c}{n_1}} =$$

$$= \frac{ncc_1(n_2 - n_1)}{n_1n_2(c_1 - c) - n(c_1n_1 - cn_2)}.$$

/245

Using the above formula to determine the osmotic value of the yeast cell, it is possible to calculate the error made in determining this value from the change in weight of the entire yeast cell instead from the change in weight of the osmotically mobile part of the cell.

If l is the osmotically mobile part of an unplasmolyzed yeast cell, l_1 the

osmotically mobile part of the plasmolyzed cell, and K the constant-weight part of the yeast cell, the result, in determining the osmotic value from the change of weight of the cell, will be

$$O = \frac{c(l_1 + K)}{l + K}.$$

In determining, from the osmotically mobile part of the cell, we have

$$O = \frac{cl_1}{l}$$

so that the error due to the change in weight of the cell will be

$$\frac{c(l_1 + K)}{l + K} - \frac{cl_1}{l} = \frac{cK(l - l_1)}{l(l + K)}.$$

Since the variable in this formula is l_1, the error will depend on this quantity; the smaller this is, the greater will be the error. In view of the fact that the osmotically mobile part of the cell decreases with increasing concentration of the plasmolytic, the error in determining the osmotic value from the change in weight of the whole cell will be greater, the greater the concentration of the solution used for the plasmolysis.

Obviously, the Kalyuzhniy method is inapplicable to bacteria, since it is extremely difficult to collect a large mass of bacterial cells.

Method of culturing on media of varying concentrations. To determine the osmotic pressure in the cell, some authors cultivated microbes on media with varying concentrations of osmotically active substances, for instance sugar, /246 sodium chloride, etc. (Boytkevich and others). The maximum concentration of the solution, at which a weak growth of the microorganism was still detectable, was taken as the index of the magnitude of the osmotic pressure in the microbial cell.

The Walter method, which is similar in principle to this method, reduces to culturing the microorganisms in Böttcher chambers, in which the relative humidity of the air is regulated by sulfuric acid.

We believe that this method does not give an exact idea as to the osmotic pressure in microbes. In fact, the adaptability of microbes to more or less concentrated solutions of various substances is well known. Therefore, when a medium with the higher concentration (let us say NaCl), is inoculated with the bacterial culture, the bacteria may remain for some time in an inactive state. However, the pressure in the medium and in the cell is gradually equalized, and the culture starts to grow. This gives the impression that the microbial cells have a high osmotic pressure. Actually, we do not measure the primary osmotic pressure characteristic for the microorganism, but the secondary osmotic pressure due to its adaptive activity.

Thus, it is only natural that this method shows the adaptability of the microbe to individual concentrations of various substances rather than the osmotic pressure characteristic for this microbe under normal conditions.

Using media with various concentrations of salt (or sugar) interesting data are obtainable, but only on the ability of the microbe to develop under certain specified conditions.

Physical methods. Various physical methods of obtaining the osmotic pressure, especially by determining the freezing point of the plant sap, are of great significance. However, for the study of microorganisms they are unsuitable, for understandable reasons.

Eschenhagen attempted to determine the osmotic pressure in the yeast cell by a cryoscopic method. He also developed a plasmolytic method and found that the former method yielded exaggerated values. Presumably, this was due to the fact that a number of substances, present in the cytoplasm, pass into the cell sap.

Vladimirov used a cryoscopic method for his bacterial studies.

The attempt by Swellengrebel to determine the osmotic pressure of the /247 yeast cell by a cryoscopic method was unsuccessful. The values obtained were considerably higher than those given by the plasmolytic method, which the author explains by passage, into the sap prepared for the experiments, of certain substances from the protoplasm, whose presence leads to an error in the determination.

In concluding our survey of the methods for determining the osmotic pressure in bacteria, it appears that the methods of the Hedin type are most interesting in this respect. Of course, the Hedin method requires proper modifications for the study of bacteria, and these we have attempted to make.

4. Recommended Method for Determining Intracellular Pressure in Bacteria

In the last Section we noted that, from our point of view, the volumetric method was highly useful for determining the intracellular pressure in bacteria.

The principal objection against the volumetric method is that, in microbes with a cell wall, it most likely will be very inaccurate. At first glance, it would seem that the presence of a membrane on the protoplast would decrease the plasticity of the cell, which would also make it difficult to determine the isotonic solution. This difficulty, however, is not substantial. We believe that the experiments by Swellengrebel, Seliber and Katznelson, as well as our own observations, given below, demonstrate that the volume of the microbial cell (bacteria and yeast) fluctuates considerably with varying concentration of the salt solution. In young cultures of microbes, the cell wall is very plastic and shrinks together with the protoplast, without leading to a detectable plasmolysis. In some cases, there is a contraction of 35% in the volume of yeast cells, with plasmolysis taking place only in occasional individuals.

Since the cell wall in aged cultures of microbes becomes less plastic, young cultures should be used in work by the volumetric method.

If a series of salt concentrations is used in the experiment and if numerical data are obtained on the change in volume of the bacterial mass, then the isotonic concentration can be found by interpolation. This was our procedure in the present investigation.

The work was usually done in the following sequence: The bacterial culture was grown either on meat-peptone broth or on agar. In the latter case, the /248 bacterial mass had to be washed with broth before the experiment. To obtain a homogeneous suspension, the bacterial flocculate was filtered through filter paper after which equal quantities of culture were poured into a slightly modified Trommsdorf tube (Fig.83) and then centrifuged for 10 min at 3000 rpm.

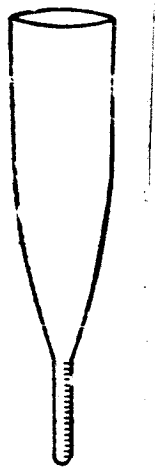

Fig.83 Tube for Determining the Osmotic Pressure in Bacteria (Trommsdorf-Type)

The bacteria settled in the capillary, and their volume was measured. The liquid from all tubes, except one (the control) was then poured off and replaced by NaCl solutions of various concentrations; the bacteria were suspended by blowing air through the capillaries, and were allowed to stand for 15 min. After this time, the tubes were again centrifuged, and the change in volume of the bacterial mass by comparison with the control was calculated. The same operations were performed in the control and experimental tubes, but instead of NaCl solution, meat-peptone broth was added.

It is obvious that hypotonic solutions caused no appreciable changes in the bacterial mass. Hypertonic solutions, however, did produce a more or less strong shrinkage of the cell volume. It was then not difficult to calculate the isotonic concentration of the salt.

It should be noted that, in culturing the bacteria for our work, we used media with very low osmotic pressures. We usually took a somewhat diluted meat-peptone broth without added sodium chloride. The osmotic pressure of such a medium, determined cryoscopically, was about 0.6 atm. Thus, the intracellular pressure obtained under our experimental conditions, was close to the turgoral pressure (under the conditions S = 0 - T; for S = 0, we have 0 = T). All test bacteria were cultured under completely identical conditions. In cases where spore cultures also exist, for example B.mycoides, we used a one-day culture, checked for the absence of spores.

In working by this method, the following must be borne in mind:

1. Above all, extreme care is required. The presence of any impurities (fibers, inorganic sediments, etc.) in the culture or salt solution will lead to incorrect results. To avoid random errors, not less than 2 - 3 parallel /249 tubes must be taken for analysis at each salt concentration.

2. The bacterial mass placed in the tube for centrifuging must be large enough to give a column of bacterial cell accumulation in the capillary, of satisfactory height. Otherwise the accuracy of the analysis is strongly impaired.

3. The conventional Trommsdorf tubes should not be used in this work, since in most cases the angle at which the wide part of the tube leads over to the capillary is too obtuse. During centrifugation, this would result in settling of a part of the bacterial mass at the point of constriction, which will ruin the analysis.

To prevent this, we modified the Trommsdorf tube. In the modified tubes, the percentage of rejected analyses was negligible in a large number of runs. The elongated end of the tube had a volume of 0.025 cc, and each division of 0.01 cm was further divided into 10 parts. We took the bacterial mass in such quantity that, after centrifuging, it occupied a volume of about 0.02 cc. The decrease in the volume of the bacterial cells was well visible, within the limits of a single division. Thus, the grossest error of our experiment could not exceed 3%.

4. It should be noted that certain bacterial cultures with colonies of slimy consistency centrifugate poorly. The method is apparently unsuitable here. This is also true for capsulogenous bacteria, since the slime surrounding the bacterial cells is able to react to the salt solutions.

To familiarize the reader with the technique employed by us, we give, as typical example, an analysis on B.herbicola (Table 70). It is easily noted that, in this case, a concentration as low as 0.1 mole of NaCl caused a contraction of the bacterial sediment.

It should be noted that here the height of the column in the control experiments did not change after the second centrifugation. This is not always the case. Sometimes the control tubes show a certain contraction, although a minor one, after the second centrifugation. In this case, in converting the

figures referring to the various salt concentrations, this contraction coefficient should be taken into account and all other calculations should make allowance for it.

The varying contraction of the same mass of bacteria in different centrifugations may be explained by the irregular operation of the centrifuge. It must also be noted, however, that most often, when the control tubes are /250 centrifuged for the second time, the height of the column is either equal to that after the first centrifugation, or differs only slightly from it.

TABLE 64

CHANGE IN VOLUME OF THE B.HERBICOLA CELL MASS, IN VARIOUS SOLUTIONS OF NACl

Salt Concentration (in Moles)	h	h_1	Ratio (in %)
Control	22.5 [1]	22.5 [2]	100
Water (distilled)	20	22	110
0.1	22	20	87.7
0.2	25	15	60
0.3	21	16	76.1
0.4	23	13	56.5
0.5	24	9	37.5

[1] h denotes the height of the sediment in MPB; h_1 the same in various concentrations of salt and other solutions after the second centrifugation.
[2] The mean values of two parallel experiments are given.

Below, we give a description of several experiments for establishing the accuracy of the results obtained by the plasmolytic and volumetric methods.

The first experiment to be presented had the object of showing the behavior of yeast cells in various concentrations of NaCl. Sodium chloride was selected as the plasmolyzing agent since almost all earlier studies on the plasmolysis of bacteria and yeasts had been made with this compound. A culture of Saccharomyces ellipsoideus, cultivated on wort agar, was used here. From this medium, the yeast was transferred with a platinum loop to test tubes filled with NaCl solutions of various concentrations. Here, the yeast cells were allowed to stand for 15 min, after which the usual preparation was made from their suspension for analysis. Under the microscope, a field with 50 - 60 cells was examined, and the percentage of cells showing appreciable plasmolysis was calculated. Cells, giving a barely perceptible marginal plasmolysis, were counted by us as plasmolyzed cells.

Together with defining plasmolysis, we also sketched the outline of 60 - 70 cells by a compass on good-quality paper. The sketched cells were then

cut out with scissors and weighed on an analytical balance. Dividing this weight by the number of cells, we obtained the relative weight of the main image of the yeast cell. From this value, the decrease in the cell volume could be estimated. In one of the subsequent experiments, we measured the length and /251 diameter of the yeast cells, on the basis of which we were able to calculate their decrease in volume.

Table 65 gives the plasmolyzability of yeast in various solutions of NaCl. It will be seen that young cells are more poorly plasmolyzed than aged cells. We also note that individual yeast cells give a clear picture of plasmolysis at various NaCl concentrations, which indicates that, even in an 0.8 mole solution of NaCl, only 43% of the aged yeast cells were plasmolyzed. At lower concentrations, the percentage of plasmolyzed cells was still lower.

TABLE 65

PLASMOLYSIS OF YEASTS IN VARIOUS SOLUTIONS OF NaCl

NaCl Concentration (in mole)	Number of Plasmolyzed Cells (in %)	
	1-Day Culture	6-Day Culture
0.4	0.0	0.0
0.5	0.0	0.0
0.6	0.0	7.0
0.7	3.5	34.0
0.8	11.0	43.0

Note. The mean results of two experiments are given.

Table 66 gives figures indicating the change in area of the yeast cell found by the above-described method. The figures are given in percent of the control. It is obvious that a considerable contraction of the cells begins at NaCl concentrations of 0.6 mole. In this case the area of a young cell decreases by an average of 21%, while the contraction, in an 0.7 mole NaCl solution, reaches 35% of the initial volume. If these figures are compared with the plasmolysis data, it will be seen that, at 0.6 mole concentration of the NaCl solution, there are no plasmolyzed cells (in the young culture) and that, at 0.7 mole of NaCl, their number reaches only 3.5%. This makes it obvious that plasmolysis takes place in a young culture only after an extensive shrinkage in cell volume. Aged cells are less plastic, which makes it understandable that they show a more pronounced plasmolysis.

It should be noted that it is not only the plasmolyzed yeast cells that /252 contract in NaCl solutions. A given sheet with such sketches can easily be arranged in order of increasing NaCl concentration, alone from the appearance of the mass of cells.

TABLE 66

CHANGE IN THE SIZE OF THE PLANE IMAGE OF A YEAST CELL IN NaCl SOLUTIONS

NaCl Concentration (in mole)	1-Day Culture	4-Day Culture	9-Day Culture
Control	100	100	100
0.2	101	97.5	98
0.4	100	95	103
0.5	99	95	104
0.6	79	90	79
0.7	64	80	86

The experiment shown in Table 66 was repeated three times, with about the same results.

These data readily show that the strong contraction of the yeast cell begins only at an NaCl concentration of 0.6 mole. It is at this instant that more or less appreciable plasmolysis takes place.

We measured the length and width of yeast cells in various NaCl solutions, in order to obtain a clear idea as to the character of the cell contraction. We measured about 70 cells in each of the solutions studied.

Table 67 gives the analytical data for this experiment. We also calculated the cell volume, on the basis of the equation of the ellipsoid, from our measurements. It is easy to see that the data are in complete agreement with the results of the preceding experiment. That is, an appreciable change in volume begins only around an NaCl concentration of 0.6 mole. It will be seen from these figures that a contraction of the cell mainly involves a decrease in width, and that reduction in length is relatively less. The yeast cell in a salt solution thus might be said to be laterally oblate. The experiment was repeated with a sugar solution. The results were entirely similar in character, so that we can omit them here.

In our further work, we had in mind a comparison of our recommended method of centrifugation with the results of the preceding tests. The yeast suspension was placed in centrifuge tubes with an elongated graduated end. After centrifugation we took the readings, the original solution was poured out, and re- /253 placed by a salt solution of different concentration. The control tube was again filled with a solution of the nutrient medium. The yeasts were suspended, and after 15 min in the new medium, were again centrifuged. The obtained reading showed the change in volume of the yeast.

As shown by the figures in Table 68, a more or less considerable decrease in volume of the yeast is again observed only in an 0.5 - 0.6 mole solution of NaCl. A contraction of less than 5% cannot be expected to give sufficiently

TABLE 67

CHANGES IN YEAST CELLS IN VARIOUS SOLUTIONS

Substrate	Average Width (in μ)	Change in Width		m	Average length of Cell (in μ)	Change in Width		m	Volume of Cell	Change in Volume (in %)
		Abs.	In %			Abs.	In %			
One-Day Culture of Yeast										
Sugar	8.41	—	—	±0.19	14.23	—	—	±0.55	526.92	—
Water	9.0	+0.59	+7	±0.24	15.27	+1.04	7.1	±0.69	647.63	—
0.4 mole NaCl	8.1	—0.31	— 3.7	±0.19	13.4	—0.83	— 5.8	±0.45	460.3	—12.7
0.5	8.57	+0.16	+ 1.9	±0.2	14.01	—0.22	— 1.5	±0.46	538.73	+ 2.2
0.6	6.74	—1.67	—19.9	±0.17	12.7	—1.53	—10.8	±0.40	302.16	—42.6
0.7	6.72	—1.69	—20.0	±0.14	13.33	—0.9	— 6.3	±0.58	315.2	—40.2
0.8	6.65	—1.76	—20.9	±0.11	13.28	—0.93	— 6.8	±0.45	307.34	—41.7
Six-Day Culture of Yeast										
Sugar	8.04	—	—	±0.22	12.27	—	—	±0.42	415.29	—
Water	8.0	+0.02	5.0	±0.26	13.44	+1.17	+9.5	±0.41	450.38	—
0.4 mole NaCl	7.77	—0.27	— 3.4	±0.21	12.69	+0.42	+3.6	±0.44	401.06	— 3.4
0.5	7.62	—0.42	— 5.2	±0.21	11.89	—0.38	—3.1	±0.49	361.53	—12.4
0.6	6.54	—1.5	—18.7	±0.15	11.11	—1.16	—9.5	±0.39	248.74	—40.1
0.7	6.22	—1.82	—22.6	±0.17	11.06	—1.21	—9.9	±0.38	224.0	—46.1
0.8	5.79	—2.25	—28.0	±0.21	11.45	—0.82	—6.7	±0.46	200.96	—51.6

reliable results. Thus, it seems that the result of the centrifugation method gives a completely reliable result. Among other things, the described experiment is not the only one performed by us. Its repetition, however, yielded nothing basically new; therefore, the additional data are omitted.

TABLE 68

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CHANGE IN VOLUME OF YEAST CELLS IN VARIOUS SOLUTIONS OF NaCl

Concentration of Solution in moles	One-Day Culture						Six-Day Culture		
	First Repetition			Second Repetition					
	h	h_1	Diff. (in %)	h	h_1	Diff. (in %)	h	h_1	Diff. (in %)
Control	23	23	0	28	28	0	20	20	0
0.4	22	22	0	28	28	0	18	18	0
0.5	22.5	20.0	9	29	27	7	18	17	6
0.6	22.5	19.0	16	29	25	14	18.5	16	14
0.7	22.5	18.0	20	29	23	21	19.5	16.7	14
0.8	22.5	17.5	22	31	24	23	16.5	14	15

On the basis of our experiments with the yeast it seemed that Fischer's explanation of the absence of plasmolysis in certain bacteria was not accurate. One might rather assume the presence of a cell wall of varying elasticity in the individual bacteria. It would then be entirely understandable that, in microorganisms in which this wall is easily shed, no visible phenomenon of plasmolysis should be observed. Experiments on B.mycoides suggested this hypothesis.

In fact, as found by us, B.mycoides does not produce plasmolysis at NaCl concentrations up to 0.8 mole. Nevertheless, if its culture is centrifuged at more or less elevated NaCl concentrations, the cell volume will contract strongly. This can be explained only by a considerable decrease in volume of the bacterial cell in NaCl solutions. To verify this proposition, we made an experiment in which we measured the width and length of B.mycoides and B.subtilis cells in various salt solutions.

The results showed distinctly that the cells of the bacilli decreased appreciably in size in hypertonic solutions. This definitely indicates that the absence of plasmolysis in bacteria is explained by the elasticity of the cell wall, rather than by the rapid equalization of internal and external concentrations, as postulated by Fischer.

The experiments by Imshenetskiy likewise established a considerable decrease in the size of the B.mycoides cell in hypertonic solutions. This decrease was directly related to the concentration of the solution.

The volume of the bacterial cell decreased in some cases to less than half its original value, and both the length and width of the cell diminished.

The sharp contraction of the bacterial cell is accompanied by morphological changes. /255 The rounded convex edges of the cell become denser on contraction of the protoplast, and the cells assume regular rectangular outlines. The interspace between the cells in the chains increases. The content of the bacterial cell ceases to be dull and homogeneous and becomes glistening, due to condensation of the cytoplasm.

According to Imshenetskiy, the separation of the protoplast into layers is observed in some of the shrunk cells in very strong NaCl solutions (about 4 moles), i.e., the typical phenomenon of plasmolysis. In the shrinking protoplast, concave zones appear, which are typical for the type of plasmolysis known as concave. The formation of convex surfaces on the shrunk protoplast is possible only when it is strongly contracted in a still more concentrated salt solution.

Imshenetskiy notes that, in water, the plasmolyzed cells are rather rapidly deplasmolyzed (within 30 - 60 sec).

Cells repeatedly plasmolyzed still retain the power of being plasmolyzed again. Thus, it may be considered that the osmotic factor, even on sharp fluctuations, possesses no pronounced bactericidal action.

The phenomenon of plasmolysis and contraction of cytoplasm, in hypertonic

solutions, can be induced only in living cells.

For finally establishing the suitability of the centrifugation method for determining the osmotic pressure in a given bacterial cell, we decided to verify its indications for various bacterial cultures.

We used B.mycoides isolated from Moscow soil. This microorganism is known to have a low osmotic pressure. For several months in succession, we cultured it on an agar medium with an addition of 0.4 mole salt solution. At first, the microbe grew only slightly on the saline medium, but then it improved although at an appreciable change in the appearance and shape of the colonies.

TABLE 69

DECREASE IN VOLUME OF THE BACTERIAL MASS IN NaCl SOLUTIONS

Concentration of Solution (in mole)	Culture on Ordinary Agar			Culture on Saline Agar		
	h	h_1	Ratio (in %)	h	h_1	Ratio (in %)
Control	4.25	4.26	100.2	4.5	4.1	91.1
0.1	4.75	3.8	80.0	6.1	5.7	98.3
0.2	3.5	3.15	87.5	4.2	3.8	90.4
0.3	5.0	4.1	82.0	4.9	4.5	91.8
0.4	5.0	3.85	77.0	4.2	4.9	90.6
0.5	5.5	3.8	69.0	5.5	4.5	81.8
0.6	6.5	3.75	57.7	4.1	3.5	85.3

After this time had elapsed, both the initial culture and the culture acclimated to saline agar were analyzed. As shown in Table 69, the bacterial culture on the saline agar showed a marked increase in intracellular pressure, which was exceptionally close to that of the surrounding medium. This was still another confirmation of the entire suitability of our method for bacteria, since it permits taking account of the shifts taking place in the bacterial cell, due to the action of the external medium.

It should be noted that Rubinshteyn and Verkhovskaya, subsequently to /256 our work, used a volumetric method to determine the permeability of the yeast cell.

Thus, from an analysis of the literature material and our own studies, we came to the conclusion that it is entirely possible to use the volumetric method for determining the osmotic pressure in bacteria.

5. Intracellular Pressure in the Geographical Races of B.Mycoides

Our study of the growth temperatures of bacteria was conducted primarily with the geographical races of B.mycoides. We also used this bacillus to study the osmotic adaptation of soil bacteria. Soils from various localities in the European part of the USSR were selected for the study, and several cultures of B.mycoides were isolated from each. After a suitable check on the purity, their intracellular pressure was determined.

The factual material is given in the following Tables. To make these Tables more compact, duplicate or triplicate determinations, which were too close together, are omitted, and only the average values after centrifuging are given.

In classifying the results, we considered a solution as weakly hypertonic if the cell shrinkage was close to 5%, relative to the control. It is difficult to consider any lesser contraction as reliably defined, with the method used.

It is interesting to note that, in accordance with the principle estab- /257
lished by us, on passage to stronger hypertonic solutions there are more or less pronounced jumps in the increased contraction of the cells, which apparently also confirms the accuracy of our conclusion.

TABLE 70

EXPERIMENT WITH B.MYCOIDES FROM IVANOVO-VOZNESENSK SOILS

Salt Concentration, Used in Centrifuging	Volume of Bacterial Cell Column after Second Centrifugation (in % of the first)		
	1st Culture	2nd Culture	Average
Control (centrifugation in broth) NaCl	97.6	96.3	97.0
0.05 mole	87.5	92.5	90.0
0.1 »	82.9	89.9	85.9
0.15 »	82.6	70.8	75.7
0.2 »	81.8	—	81.8

[1] The first centrifuging was done in broth, the second at various concentrations of NaCl.

The data given below are arranged by regions, beginning with the northerly and gradually passing to the south.

Experiments with cultures of B.mycoides from soils of Ivanovo-Voznesensk vicinity. The results of the experiments with two cultures from Ivanovo-Voznesensk soils are given in Table 70.

It follows from these data that a salt solution close to 0.05 mole may be considered weakly hypertonic for the Ivanovo-Voznesensk cultures of B.mycoides. In such an NaCl solution, the contraction relative to the control is on the average around 7%. In our experiments, this is the smallest value found for a hypertonic solution. Comparing the climatic data of the Ivanovo-Voznesensk region with the others in our study, one could say that this is the region with the least heat and the most moisture. Hence, it is obvious that the intracellular pressure of soil bacteria should be very low in this case, which is in fact observed.

Cultures of B.mycoides from soils of the Moscow vicinity. Table 71 gives the data obtained for three cultures of B.mycoides isolated from Moscow soil. It is clear that the intracellular pressure for these bacteria is rather low. A solution less concentrated than 0.1 mole of NaCl is isotonic with it. Un- /258 fortunately, we used no 0.05 mole NaCl concentration in this experiment. However, since an 0.1 mole solution of NaCl is strongly hypertonic, there is reason to believe that the intracellular pressure is not higher for the Moscow cultures of B.mycoides than for cultures from soils of the Ivanovo-Voznesensk region. Assuming that the climate has an influence on the physiological properties of soil bacteria, a certain regularity can be noted in this, seeing that the climatic data of Moscow and Ivanovo-Voznesensk are identical.

TABLE 71

EXPERIMENT WITH MOSCOW CULTURES OF B.MYCOIDES

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)			
	1st Culture	2nd Culture	3rd Culture	Average
Control	100	100	100	100
0.1	81.2	80.0	84.4	81.4
0.2	85.7	90.9	66.6	80.8
0.3	75.0	82.0	83.3	80.1
0.4	80.0	77.0	—	78.5
0.5	76.3	69.0	84.4	76.6
0.6	71.4	57.6	76.6	68.5

Experiments with cultures of B.mycoides from soils of the Menzelinsk vicinity. The Menzelinsk cultures of B.mycoides, as shown in Table 72, begin to show a marked decrease in cell volume at an NaCl concentration of about 0.1 mole, so that this solution can be considered hypertonic in our case. For the Menzelinsk region, the amount of heat received during the warm period of the year is very close to the Ivanovo-Voznesensk conditions, except that there is considerably less rainfall. Most likely, this shows in an increased intracellular pressure for B.mycoides cultures in this region.

Experiments with B.mycoides from soils of the Kalach vicinity. B.mycoides

from the soils of Kalach show a more or less pronounced decrease in volume on centrifuging in NaCl of 0.15 - 0.2 mole concentration.

Solar radiation at Kalach is more intense than at the preceding place, while the rainfall is about the same. This makes the climate drier, which /259 shows in the intracellular pressure of the B.mycoides from this region.

TABLE 72

EXPERIMENT WITH MENZELINSK CULTURES OF B.MYCOIDES

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)				
	1st Culture	2nd Culture	3rd Culture	4th Culture	Average
Control	90.0	93.7	95.0	99.1	94.4
0.05	—	—	—	94.6	94.6
0.1	83.7	83.3	89.5	53.9	87.6
0.15	82.0	83.3	—	94.8	86.7
0.2	73.0	77.8	85.0	—	78.6

Region of Popelyansk. Popelyansk has almost about the same climatic indices as Kalach.

TABLE 73

EXPERIMENTS WITH CULTURES OF B.MYCOIDES IN SOILS FROM THE VICINITY OF THE CITY OF KALACH

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)			
	1st Culture	2nd Culture	3rd Culture	Average
Control	97.4	95.8	94.4	95.9
0.1	96.3	91.7	91.7	93.2
0.15	95.6	90.2	86.6	90.8
0.2	90.9	89.8	87.2	89.3
0.25	87.0	66.0	—	86.8
0.3	86.1	85.8	—	86.0

Here, however, the intracellular pressure for B.mycoides is appreciably higher. Thus, a more or less extensive shrinkage in cell volume is observed

only at an NaCl concentration of about 0.25 mole. A pattern of this type is observed in all three cultures of B.mycoides studied, thus excluding the possibility of an error. The fact that the intracellular pressure was higher for /260 the Popelyansk region than had been assumed, may perhaps be explained by certain factors connected with its climate and not taken into account by us.

TABLE 74

EXPERIMENTS WITH CULTURES OF B.MYCOIDES FROM THE NEIGHBORHOOD OF POPELYANSK

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)			
	1st Culture	2nd Culture	3rd Culture	Average
Control	97.5	100	97.5	98.3
0.2	100	98.9	97.4	98.8
0.25	91.9	93.2	90.6	91.9
0.3	89.1	94.1	91.0	91.4
0.4	88.9	94.0	—	91.5

Region of City of Ryl'sk. In the Ryl'sk region, which is somewhat colder and damper than the above zones, the intracellular pressure of B.mycoides declines. In this case, we consider an 0.15 mole solution as weakly hypertonic, since a detectable contraction in volume of the bacterial mass is present here.

TABLE 75

EXPERIMENTS WITH B.MYCOIDES FROM SOILS OF THE RYL'SK REGION

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)		
	1st Culture	2nd Culture	Average
Control	95.3	95.3	95.3
0.1	94.0	91.1	92.6
0.15	88.9	87.7	88.8
0.4	83.8	83.7	83.5

Region of Belaya Tserkva. Table 76 gives the result for two B.mycoides cultures, isolated from soils of the Belaya Tserkva region. These more souther-

ly places show a certain increase in intracellular pressure. A concentration of about 0.25 mole is isotonic to them.

TABLE 76

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EXPERIMENTS WITH B.MYCOIDES FROM SOILS OF THE REGION OF BELAYA TSERKVA

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)		
	1st Culture	2nd Culture	Average
Control	100	100	100
0.1	102.8	100	101.4
0.2	100	100	100
0.25	92.3	93.2	92.8
0.5	88.2	83.3	85.7
0.7	88.2	87.8	88.0

Region of Kirsanov. We now give data for two B.mycoides cultures of the Kirsanov region, which is somewhat colder than the last one. Here an entirely reliable contraction in the volume of the bacterial mass is noted at about an 0.1 mole NaCl solution.

TABLE 77

EXPERIMENTS WITH B.MYCOIDES FROM SOILS OF THE KIRSANOV REGION

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)		
	1st Culture	2nd Culture	Average
Control	100	90.7	95.4
0.05	100	—	100
0.1	95.9	85.0	90.5
0.15	95.3	87.7	91.5
0.2	93.0	83.0	87.5

Donets region. B.mycoides for these experiments was isolated from soils near Ambrosiyevka.

The Donets Basin which has little moisture, also places its stamp on the intracellular pressure of B.mycoides. Here we consider an NaCl concentration close to 0.2 mole as weakly hypertonic. An NaCl concentration of 0.2 mole, for example, gives a 16% contraction in volume for the Ivanovo-Voznesensk culture,

but here it induces only a minor contraction of the bacterial column.

Region of Dolinskaya Station (North Caucasus). The somewhat moister /262 region of Dolinskaya Station does not substantially change the intracellular pressure by comparison with the preceding place.

TABLE 78

EXPERIMENTS WITH B.MYCOIDES FROM DONBAS SOILS

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)		
	1st Culture	2nd Culture	Average
Control	98.5	96.9	97.7
0.2	95.6	94.3	94.9
0.25	92.4	88.9	90.5
0.3	91.2	88.4	89.8
0.4	—	87.9	87.9

TABLE 79

EXPERIMENTS WITH B.MYCOIDES FROM SOILS OF DOLINSKAYA STATION

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)			
	1st Culture	2nd Culture	3rd Culture	Average
Control	95.0	95.4	92.3	94.2
0.2	91.0	95.1	87.5	91.2
0.25	—	—	78.6	78.6
0.3	87.6	85.5	78.5	83.9

It is true that there is a barely perceptible decrease of this pressure. Thus, in 0.25 and 0.3 mole NaCl solutions, we observe a greater contraction of cell volume than in the Donbas cultures of B.mycoides but this difference cannot be translated into numerical values, because of the imperfection of our method. The weakly hypertonic NaCl solution in this case is close to 0.2 mole.

Melitopol' region. Table 80 gives the data for the Melitopol' culture of B.mycoides. The Melitopol' region, which is very arid, increases the intra-

cellular pressure of B.mycoides. Here we note a marked contraction in volume /263 of the bacterial mass only at an 0.25 mole concentration of NaCl. We adopt this concentration as that of the weakly hypertonic solution for the Melitopol' cultures of B.mycoides.

TABLE 80

EXPERIMENTS WITH B.MYCOIDES FROM MELITOPOL' SOILS

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)
Control	96.1
0.2	96.0
0.25	92.1
0.3	87.5
0.35	64.7

Pavlovsk region (North Caucasus). The Pavlovsk region of North Caucasus may be considered somewhat moister than the Donets and Melitopol' regions. However, the pressure in the B.mycoides cultures of this region is higher than that observed in the Melitopol' region. The hypertonic NaCl solution here is around 0.35 mole NaCl. This deviation cannot be considered accidental, since the races of B.mycoides in the Krasnodar area also indicated a high intracellular pressure.

TABLE 81

EXPERIMENTS ON NORTH CAUCASUS CULTURES OF B.MYCOIDES

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)			
	1st Culture	2nd Culture	3rd Culture	Average
Control	98.9	93.3	98.4	96.9
0.1	97.7	93.3	—	95.5
0.2	95.5	100.0	96.6	97.4
0.3	93.3	97.7	95.4	95.1
0.4	81.8	82.1	87.4	83.8

Experiments with Krasnodar cultures of B.mycoides. Table 82 gives /264
the results for the Krasnodar cultures of B.mycoides. Here we find a still higher intracellular pressure than in all of the other cultures studied. A weakly hypertonic NaCl solution would be more concentrated than 0.4 mole.

TABLE 82

EXPERIMENTS ON KRASNODAR CULTURES OF B.MYCOIDES

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)		
	1st Culture	2nd Culture	Average
Control	100.1	100	100
0.1	102.4	102.9	102.6
0.2	100	94.9	97.4
0.3	103.3	104.0	103.6
0.4	100	100	100.0
0.5	96.9	93.3	95.4
0.6	93.3	85.7	89.5
0.8	80.0	—	80.0

Experiment with cultures of B.mycoides from Kherson soils. Table 83 gives the data for two B.mycoides cultures isolated from soils of the Kherson Experimental Station. Their intracellular pressure is fairly high, and obviously the weakly hypertonic NaCl concentration would be close to 0.4 mole.

TABLE 83

EXPERIMENTS ON KHERSON CULTURES OF B.MYCOIDES

Concentration of NaCl (in mole)	Volume of Bacterial Cell Column after Second Centrifugation (in % of First)		
	1st Culture	2nd Culture	Average
Control	100	93.5	96.7
0.1	101.0	97.7	98.8
0.2	101.4	102.0	101.7
0.3	100.0	100.0	100.0
0.4	76.3	93.0	87.6
0.5	73.3	91.1	82.2
0.6	73.3	88.8	81.0

In concluding this Section, Table 84 summarizes the data on the intracellular pressure of various cultures of B.mycoides. The fact of the existence /265
of a higher intracellular pressure in the southern races than in the northern

is the most striking feature here. Of course, this can be explained by the fact that the southern soils are physiologically a drier medium than the northern.

TABLE 84

SUMMARY OF EXPERIMENTAL RESULTS ON THE INTRACELLULAR PRESSURE OF GEOGRAPHICAL B.MYCOIDES RACES

Longitude (in deg.)	Latitude (in deg.)	Site of Sampling (District)	Weakly Hypertonic NaCl Solution (in mole)		Intracellular Pressure (in atm)	
41	57	Ivanovo-Voznesensk	about	0.05	about	1.8
38	56	Moscow	»	0.05	»	1.8
53	56	Menzelinsk	»	0.1	»	3.6
41	50.5	Kalach	»	0.15—0.2	»	5.4—7.2
30	50	Popelyansk	»	0.2—0.25	»	7.2—9.0
35	51.5	Ryl'sk	»	0.15	»	5.4
30	50	Belaya Tserkva	»	0.25—0.3	»	9.0—10.8
43	53	Kirsanov	»	0.1	»	3.6
38.5	48	Donbas	»	0.25	»	9.0
33	48	Dolinskaya	»	0.2	»	7.2
35	47	Melitopol'	»	0.25	»	9.0
40	46	Pavlovsk	»	0.35	»	12.6
39	45	Krasnodar	»	0.4—0.45	»	14.4—16.2
32	46.5	Kherson	»	0.4	»	14.4

It should be noted that the intracellular pressure of B.mycoides persistently maintains its own characteristic value. We cultivated individual cultures of B.mycoides on MPA for a long time and then again determined the intracellular pressure. The latter remained constant in both northern and southern cultures.

After the experiments with B.mycoides, we decided to verify the reaction to climate with respect to the change in the intracellular pressure of other microbes. First, as an easily identified microbe, we took Azotobacter chroococcum isolated from various soils on silica gel. It is true (as already noted) that Azotobacter, in view of its capsule formation, is a poor object for work by the proposed method. Nevertheless, in order to obtain even approximate figures confirming the general tendency, we decided to make determinations on this organism as well. We selected a young culture of Azotobacter for centrifugation.

The intracellular pressure was determined by the usual method. Table 85 gives the results. /266

Quite obviously, the same pattern as that given by B.mycoides is repeated here. The Moscow race of Azotobacter has an osmotic cell pressure of less than 3.5 atm, while that of the Poltava race is about 7 - 9 atm, that of the still more southerly Kherson race, 12 atm, and, finally, that of the Krasnodar race,

18 atm. It will be noted that, in the same localities, entirely different microbes have about the same intracellular pressure.

TABLE 85

OSMOTIC PRESSURE IN THE CELL OF SOIL BACTERIA

Site of Sampling (Vicinity)	Mixed Culture		Azotobacter Chroococcum	
	Weakly Hypertonic NaCl Solution (in mole)	Intracellular Pressure (in atm)	Weakly Hypertonic NaCl Solution (in mole)	Intracellular Pressure (in atm)
Moscow . . .	0.1	3.6	0.1	3.6
Poltava . .	—	—	0.2—0.25	7.6—9.4
Mariupol' . . .	about 0.2	about 7.2	—	—
Kherson . . .	» 0.3	» 10.8	about 0.35	about 12.6
Krasnodar . . .	» 0.4	» 14.4	» 0.5	» 18.0

We followed this by experiments with mixed cultures. Since the variation in intracellular pressure of the microbes presumably is of the same type, regardless of their specific difference, it was postulated that the mixed cultures of soil bacteria would give no less significant variations than pure cultures.

To obtain mixed cultures, flasks with broth were inoculated with mixes of various soils and were then kept in the incubator for two days. After multiplication, the bacterial culture was passed through a filter and then used for the experiments.

Enriched cultures of this type were prepared from a number of soils. Table 85 gives the results.

It will be seen that our hypothesis was fully confirmed, and that the absolute figures, expressing the intracellular pressure for a large variety of bacterial species in the mixed culture, were very close to those obtained for the above pure cultures.

In conclusion, it should be mentioned that Sushkina fully confirmed the existence of a higher osmotic pressure in the southern races of Azotobacter.

6. Relation between Intracellular Pressure of B.mycoides and Climatic Indices

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Table 86 gives the localities from which the B.mycoides cultures were obtained. Together with the geographical point we also give the climatic data for any indices that could have affected the intracellular pressure in B.mycoides. We consider that these indices were: 1) the total heat received during the period that the air temperature was above 10° C; and 2) the rainfall in mm

during this period.

Based on these indices, we felt justified to confine the investigation to the hot period of the year, since the physiological traits of microbes can be affected only during this time of year.

Of course, the factors selected here are not necessarily optimum for a correlation with the data on intracellular pressure, but for lack of more exact data characterizing the aridity of a given locality - which is the main factor influencing the intracellular pressure of bacteria - we were forced to use these bases.

The indications of total heat had to be given a correction for humidity. This correction, while accurate in principle, was unable to modify the ratio

$$\frac{\text{thermal regime of locality}}{\text{intracellular pressure of bacteria}}$$

to a significant extent, since the differences in moisture between the selected regions must be more or less negligible by comparison with the absolutely greater quantitative expressions of the temperature factor. Further than that, there were almost no meterological data to characterize the aridity of the climate. However, in our work we also used arbitrary indices to characterize the aridity of the climate, calculated by the Selyaninov principle. The latter author noted that the cumulative temperature for a given period, divided by 10, agrees rather well with the quantity of moisture evaporated in the shade at the given locality (during the hot season of the year). The ratio of rainfall to evaporation also yields an index of the influx of rain. At an index equal to unity, the income of moisture equals its outgo; for an index less than unity, there will be a shortage of water, which will be greater the smaller the index.

In his calculations, for the growth period of the higher plants, Selyaninov used only the three summer months, June, July, and August, considering that the climatic data of this period determine the harvest. This "Selyaninov index" /268 should perhaps have been calculated differently for the life of soil bacteria, namely, by using the period during which bacteria can actively multiply in the soil; however, in practice this would be extremely difficult, because of the lack of adequate meteorological data.

The diagrams in Figs.84 and 85 give a graphic representation of the relationship between the intracellular pressure in B.mycoides and the climatic features. Thus, Fig.84 compares these pressures with the mean annual air temperature. It will be seen that, despite a certain arbitrariness of this comparison, the general regularity of the resultant line is entirely satisfactory. Certain deviations, as (for instance) in the Menzelinsk cultures, are entirely understandable, since the factor of aridity was not taken into account here. Menzelinsk, however, lies in a belt less well supplied with moisture than Moscow.

The line of the diagram in Fig.84 is very close to a straight line. Thus the adaptation of bacteria, as far as the regulation of the intracellular

TABLE 86

CLIMATIC DATA FOR LOCALITIES OF SOIL SAMPLING, FOR ISOLATION OF B.MYCOIDES

District of Sampling	Mean Annual Air Temperature	Cumulative Temperature for the Period with Temperatures above 10°C	Rainfall for May-June	Index of Aridity
Ivanovo-Voznesensk	3.3	about 1 900	175	1.26
Moscow	4.3	» 2 100	196	1.36
Menzelinsk	2.2	» 2 000	170	1.22
Kirsanov	4.6	» 2 300	159	1.03
Ryl'sk	5.2	» 2 500	232	1.08
Kalach	6.8	» 2 800	179	1.09
Popelyansk	6.6	» 2 600	289	0.9
Belaya Tserkva	6.9	» 2 900	322	0.89
Dolinskaya	7.5	» 3 300	165	0.92
Ambrosiyevka (Donbas)	7.4	» 2 800	147	0.87
Melitopol'	9.6	» 3 300	143	0.87
Kherson	10.1	» 3 300	121	0.67
Pavlovsk (North Caucasus)	9.5	» 3 300	ок. 160	ок. 0.9
Krasnodar	11.1	» 3 500	171	0.93

pressure goes, is expressed by the same law as their adaptation to the tem- /269
perature.

A similar law is obtained for the relationship between the osmotic pressure of the B.mycoides cell and the cumulative temperature for the hot period of the year (Fig.85).

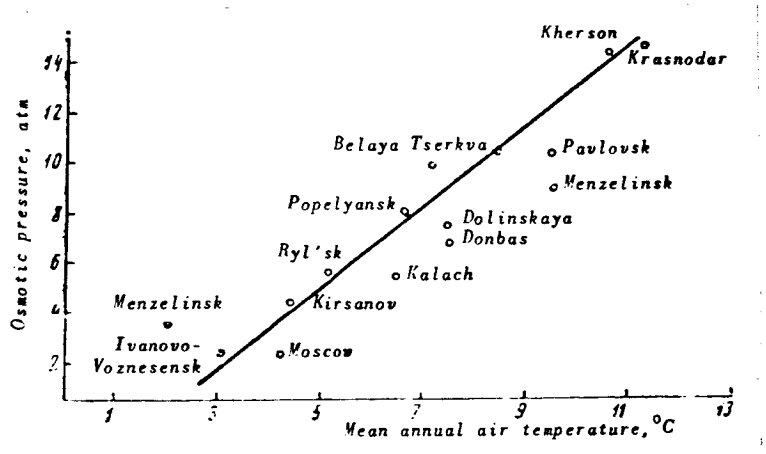

Fig.84 Relation between the Osmotic Intracellular Pressure of the B.Mycoides Cell and the Mean Annual Atmospheric Pressure

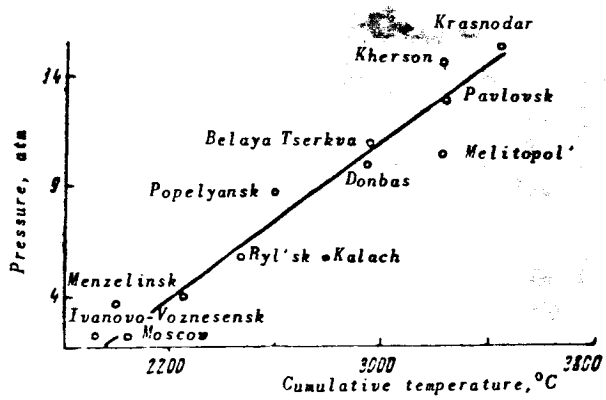

Fig.85 Relation between Cumulative Temperature for Hot Period and Intracellular Pressure in B.Mycoides

Despite the highly arbitrary nature of the Selyaninov index, there is still a linear relationship between it and the intracellular pressure of B.mycoides.

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Thus, this work permits the following conclusion:

1. The intracellular pressure in the individual cultures of B.mycoides varies over a wide range with the climatic conditions of the soil. A similar adaptability is noted in other soil bacteria.

2. In the present work, B.mycoides cultures, from a number of different localities in the USSR, were analyzed. The data on the intracellular pressure of B.mycoides correlate rather well with the indices on the thermal regime of the places studied, and the corresponding relation can be expressed by the equation of a straight line.

CHAPTER IX /271

THE PROCESS OF GENESIS OF ADAPTIVE FORMS IN BACTERIA

As noted in the introductory Chapter, Darwin repeatedly speaks of the regular variation of organisms as a function of geographical factors.

In Darwin's opinion, the geographic series in living organisms arise as a consequence of the struggle for existence. In his works, he foresaw the inheritance of ecological and geographical traits of organisms, acquired as a result of selection.

Geographical variations are connected with ecological factors, i.e., they are essentially adaptive. While not always very distinctly shown in higher organisms, this proposition finds clear-cut confirmation in the example of microorganisms, as will be seen from the material presented. In his Origin of Species, Darwin writes: "... Each organism manifests a tendency to become more perfected with respect to the conditions surrounding it".

New forms arise with varying conditions of existence only as a result of selection. "Where there is no selection, sharply differing races are never and nowhere formed".

The adaptive character of racial traits is a powerful argument in favor of the selective origin of races.

Anti-Darwinists have attempted to answer the question as to the origin of geographical forms without using the theory of selection, considering that the prolonged action of the environment makes traits hereditary. This proposition is in complete contradiction to the basic postulate of the evolutionary view.

Darwin stated that the environment is directly able to modify the traits of plants and animals, but that these modifications are superficial and without serious significance for evolution. Climatic factors, no matter how strong, act mostly indirectly (by selection), favoring some form and having a lethal /272 effect on others.

Not believing that adaptations, including geographical adaptations as a result of the direct influence of the environment on the organism were possible, Darwin wrote: "If, for example, it is desired to modify a plant so as to adapt it for transfer from a waterless place to a wet place, there is no reason to assume that the desired kind of variation would appear more frequently if the parent plant were grown in a somewhat moister place than usual. All the same, whether the place be unusually dry or damp, eventually variations that slightly adapt the plant to the directly opposite mode of life will appear by chance, as we have reason to assume from what we know of other cases". It follows from this that Darwin considered natural selection to be responsible for the path and form of the evolutionary process. "Logically arguing", Timiryazev wrote, "we should realize that the variability due to environment is in itself indif-

ferent. Variations may be useful to the organism, indifferent, or directly harmful. The imprint of adaptation, of usefulness, is made not by the physical process of variation, but by the subsequent process of elimination of the useless, i.e., by selection".

The materials for evolution yield mutations, which are common in the living world. In any natural population of a certain species of living organism, there is always an enormous hidden reserve of ready mutations which may yield the origin of new forms with every variation in environment. Natural selection discloses the forms which are consonant with a given external environment.

A number of investigators (Fischer, Holden, Wright, Dubinin, and others) have shown that hereditary variations, occurring singly, may multiply in a population and constitute the basis for the evolutionary process. Even the smallest deviations may be of selectional significance.

The new form may rapidly displace the old and less adapted form.

This raises the question whether evolution can be explained, primarily or exclusively, by the appearance of sudden mutations produced by chance shifts in the hereditary substance of the cell. Darwin did not go into detail on the causes responsible for the variation of the hereditary traits of the organism.

Timiryazev, Michurin, and Lysenko, later developing Darwin's doctrine, pointed out that the living conditions influence the hereditary endowment of living beings. This proposition permits active interference with heredity /273 by the creation of definite conditions of life for the organisms, and by subsequent selection. Obviously, also in a natural environment, the determining environmental conditions lead to an intensification of the useful traits in the progeny and to an elimination of the undesirable traits.

In the introductory Chapter of this book, we mentioned that Darwin's works also contained isolated references which showed that he attached great importance to the influence of living conditions on the hereditary endowment of living beings.

Engels, in his "Dialectics of Nature" emphasized this proposition, noting: "The thought of the gradual transformation of the earth's surface and of all conditions of life on it has led directly to the doctrine of the constant transformation of organisms and of their adaptation to a varying environment, and thus resulted in the doctrine of the 'variability of species'".

The variability of microbes, especially under laboratory conditions, under the influence of widely differing factors, has been studied in numerous investigations carried on in other countries and in the USSR. The results of these studies have been summarized in the article by Imshenetskiy published in 1946 (Uspekhi sovr. biol., Vol.21, No.1). Krasil'nikov (Zhurnal obshchey biol., Vol.4, 1943) has collected material on the individual variability of microbes.

Many investigators have the impression that the properties of bacteria vary readily, which often led to their traits to be contrasted with those of higher organisms.

Our own work on the temperature and osmotic adaptation of soil microflora makes it impossible for us to adhere to this view. We had sufficient opportunity to demonstrate the relative stability of the hereditary traits of bacteria. Microorganisms in general, and bacteria in particular, have very short individual lives. The isolated deviant forms that occur in their cultures can therefore multiply within a very short time.

Thus, the readiness with which bacteria become adapted should be interpreted as the relative rapidity of the development of new forms. This may possibly require as many generations as in higher beings. However, their rapid succession does give the experimenter the erroneous idea of mass variability of bacteria under the influence of environmental conditions.

In the material to be presented below, we desire to show that the formation of races among microorganisms, exactly as is the case among higher organisms, is based on the selection of mutations whose traits are useful in connection with a certain external environment. /274

However, from certain examples, we note that the modified environmental conditions lead to the appearance of deviant forms, not encountered in a population grown in the old environment. This clearly depicts the creative role of selection, and prevents one from degrading its function to the level of a strainer that removes forms not suited to the new situation as it develops.

Since, in our work, we were concerned with the temperature and osmotic properties of the geographical races of soil bacteria (mainly of B.mycoides), we will also discuss them in this Section.

Our experimental work was performed on pure cultures of B.mycoides. At first, we wanted to determine to what extent the individual geographical races of B.mycoides can tolerate an increase in osmotic pressure of the environment. The following cultures of B.mycoides were taken for the work:

Moscow, with an osmotic pressure of about 2 atm;
Khar'kov, with an osmotic pressure of about 9 atm;
Krasnodar, with an osmotic pressure of about 15 atm.

These bacteria were cultured on MPA and MPB, containing various amounts of sodium chloride.

Table 87 gives the results. As indicated there, all cultures of B.mycoides selected for this experiment are able to grow in solutions with an osmotic pressure higher than characteristic for their cell. For example, the Moscow culture with an intracellular pressure near 2.0 atm, will grow on MPB containing 0.6 mole NaCl solution.

Second, it is obvious that, on liquid and solid nutrient media, the bacteria show a dissimilar tolerance to the presence of high NaCl concentrations. They will grow better in a saline liquid medium than in a solid medium. The Moscow culture did not grow on MPA with 0.5 mole NaCl, but did show growth on MPB, even if the medium had contained an 0.6 mole solution of NaCl. At first glance, this situation may be explained by the greater ease with which the cell-

TABLE 87

GROWTH OF B.MYCOIDES ON SALINE MEDIA

NaCl Concentration (in mole)	Growth of B.Mycoides on Meat-Peptone Agar						Growth of B.Mycoides on Meat-Peptone Broth					
	Moscow Race		Khar'kov Race		Krasnodar Race		Inoculation with a Thick Suspension of the Following Cultures			Inoculation with a Weak Suspension of the Following Cultures		
	I	II	I	II	I	II	Moscow	Khar'kov	Krasnodar	Moscow	Khar'kov	Krasnodar
Control	++++	35.0	++++	45.0	++++	43.0	++++	++++	++++	++++	++++	++++
0.1	++	6.0	+++	18.0	++++	25.0	+++	++	+++	+++	+++	+++
0.2	++	6.0	+++	18.0	+++	17.0	++++	++++	+++	+++	+++	+++
0.3	++	3.0	++	9.0	++	7.0	++++	+++	+++	+++	+++	+++
0.4	+	2.5	+	1.5	+	4.0	+++	++++	+++	++	++	++
0.5	0	0.0	+	weak	+	1.5	+++	+++	++++	+	+	+
0.6	0	0.0	+	"	+	weak	+++	+++	+++	+	+	+

Note. 1. The measurements were made on the second day after inoculation of the medium.
2. Signs in first column: ++++ luxuriant growth; +++ good growth; ++ satisfactory growth; + weak growth; 0 no growth.
3. Column II shows the mean size of the developed colonies on the medium (in mm).

solution system attains equilibrium in a liquid medium.

The individual cultures of B.mycoides, in general, show almost no difference in their growth on saline MPB. The picture is different for the growth of geographical races of B.mycoides on saline MPA. Here, the B.mycoides from Krasnodar soil grow best on a medium with high NaCl concentration. The Moscow culture stops growing in a less concentrated solution; in the presence of as little as 0.1 mole of NaCl, the size of its colonies reduces sharply while the southern races develop well on such a medium. /276

Third, it is particularly significant that the quantitative aspect of the inoculation obviously plays a major role in the tolerance of a culture for a high NaCl concentration. For example, in saline MPB with higher amounts of NaCl, the growth of B.mycoides was better when the medium was inoculated with a thick bacterial suspension. In this case, to all appearances, more halotolerant cells from within the bacterial population passed into the medium. This assumption also would explain the fact that the cultures showed a greater tolerance to high NaCl amounts in the broth. If only a single halotolerant cell passes into a liquid medium, it will cause clouding of the medium on its proliferation. However, in culturing on a saline agar medium, isolated growing cells cannot mask the depressive state of the culture as a whole. Thus, the resultant data indicate that only a few cells of the culture can tolerate elevated concentrations of NaCl.

To verify the existence of halotolerant cells in the population, an experiment was performed with the following setup:

Agar containing various amounts of NaCl was poured into Petri dishes. After hardening, a smear of a B.mycoides MPA culture (Khar'kov race) was inoculated on its surface with a platinum loop. The material for inoculating the agar was of different density in the individual cases. In the first series, we used a thick suspension of bacteria which, in the following series, was diluted with sterile water to several times its volume. The weakest dilution (18 times) showed considerable cloudiness, plainly apparent to the naked eye. A one-day B.mycoides culture was used in this experiment. The growth of the bacteria was estimated on the second day, after infection of the medium (for the results, see Table 88).

The conclusions drawn from this experiment were entirely definite. Where inoculation along the streak was made with a sufficiently thick bacterial suspension, we observed a continuous and abundant growth of B.mycoides, up to an 0.3 mole concentration of NaCl on the medium. At higher NaCl concentrations, bacterial growth in separate islands was noted. The only explanation would be that only some rather than all of the bacteria placed on the nutrient medium germinated. Evidently, not all bacterial cells of B.mycoides have equal tolerance to higher NaCl concentrations.

On a solid medium, this development is easy to define while, on a liquid medium, it is not detectable since the salt-resistant cells multiply rapidly and give the impression of a luxuriant growth of the culture as a whole.

This readily explains the finding that, after diluting the original culture

18 times, no bacterial growth at 0.4 mole NaCl was observed. Here the dilution evidently was sufficient to prevent even a single cell of NaCl-adaptable B.mycoides from getting onto the platinum loop used for transfer to the agar. Insufficient inoculation is not in question here, since luxuriant growth along the entire streak was noted on ordinary agar.

TABLE 88 /277

GROWTH OF B.MYCOIDES ON SALINE MPA

Medium	Dilution of Bacterial Suspension				
	None	2 Times	3 Times	6 Times	18 Times
Ordinary agar	Abundant growth along streak	Abundant growth along streak	Abundant growth along streak	Abundant growth along streak	Abundant growth along streak
Agar with 0.1 mole of NaCl	Same	Same	Same	Same	Same
Agar with 0.2 mole of NaCl	Same	Same	Same	Same	Same
Agar with 0.3 mole of NaCl	Same	Same	Same	Same	Growth in patches
Agar with 0.4 mole of NaCl	Growth in patches	Growth in patches	Growth in patches	Growth in patches	No growth
Agar with 0.5 mole of NaCl	Same	Same	Same	No growth	Same

Thus, the ordinary cells of the culture also comprised some halotolerant individuals which, of course, must be considered mutations. We will demonstrate below that, from such forms, races of B.mycoides distinguished by elevated osmotic cell pressure can be obtained by selection. This trait is transmitted to the progeny.

Evidently, it is also so in nature that such forms, deviating from the normal under varying environmental conditions, will be the starting points for the development of races.

The literature contains references to the existence, in bacterial cultures, of cells resistant to various physical and chemical factors. Our own observations thus confirm facts already described by other authors.

To establish the percentage of cells "resistant" and "non-resistant" to the action of NaCl in individual cultures of B.mycoides, the following experiments were made: /278

An aqueous bacterial suspension was prepared from fresh cultures of B.mycoides, and agar media with various amounts of salt were inoculated with equal amounts of this suspension. MPA was inoculated in the melted state, with B.mycoides. For several days, the number of colonies formed were counted on each medium. In this way, we were able to establish the inhibiting action of various amounts of NaCl on bacterial growth. Table 89 gives the results of this experiment, made with several B.mycoides cultures. Observations were taken 1 and 4 days after inoculation. The Table gives the data in percent of the number of colonies grown on ordinary agar, within 24 hours after inoculation. The number of bacteria on the medium usually fluctuated in the range of 100 per dish.

TABLE 89

EFFECT OF VARIOUS AMOUNTS OF NaCl ON THE GERMINATION OF CELLS OF B.MYCOIDES

NaCl Concentration (in mole)	Moscow Race		Khar'kov Race		Krasnodar Race	
	1st Day	4th Day	1st Day	4th Day	1st Day	4th Day
—	100[1]	—	100	—	100	—
0.1	107.4	—	91.3	—	51.0	—
0.2	2.4	106	[illegible]	[illegible]	[illegible]	140
0.3	0.0	52	0.0	80.0	0.0	110.0
0.4	0.0	0.0	0.0	120.0	0.0	117.0
0.5	0.0	0.0	0.0	0.6[2]	0.0	0.8
0.6	0.0	0.0	0.0	0.0	0.0	0.0

[1] Number of bacteria grown on ordinary agar within 24 hours after inoculation, taken as 100.
[2] Very small colonies.

This experiment shows that most of the bacteria were able to grow only at a relatively low NaCl concentration in the nutrient.

Thus, assuming that the isotonic concentration of NaCl for the Moscow race of B.mycoides is 0.05 mole NaCl, it is obvious that only a few cells of this organism will grow rapidly on a medium with over 0.2 mole of NaCl.

For obvious reasons, the Khar'kov and Krasnodar races tolerated higher /279

NaCl concentrations, but even here the growth was strongly inhibited at a slightly higher concentration.

The percentage of cells that will grow at 0.6 mole of NaCl in the medium is negligible. In any case, in our experiments in which MPA was inoculated with about 100 B.mycoides cells on a medium with 0.6 mole of NaCl, there was not a single proliferated colony after four days. Nevertheless, such tolerant cells must exist since, on MPB with 0.6 mole of NaCl inoculated with a richer bacterial suspension, all cultures studied by us gave satisfactory growth. Of course, development in this case was due to multiplication of the few cells that had become adapted to a high NaCl concentration.

In our subsequent work, we attempted to obtain a culture with a higher intracellular pressure from the Moscow B.mycoides. With this object in mind, we cultured a thick mix of bacterial cells on MPA with 0.4 mole of NaCl. In several days, discrete colonies of B.mycoides appeared on the agar medium, which were then transferred to test tubes containing saline agar. After prolonged cultivation of the obtained culture on the saline medium, it was transferred to ordinary MPA and the intracellular pressure was determined volumetrically. Simultaneously, a similar experiment was made on the initial culture of B.mycoides.

This experiment showed that, if the Moscow race of B.mycoides had an intracellular pressure isotonic to an 0.05 mole NaCl solution, then the newly developed race showed an appreciable contraction of the bacterial column at an NaCl concentration of about 0.4 mole.

Subsequently, during a number of transfer cultures of the new race of B.mycoides on ordinary agar, the elevated intracellular pressure was persistently maintained.

This experiment showed clearly that individual mutations of bacteria (in this case, changed osmotic pressure), in the presence of selection, serve as the nucleus for the formation of races.

The above proposition on the osmotic phenomena remained entirely valid for shifts in the growth temperatures, although adaptation to the latter factor does not take place as readily.

In the following Section, we will attempt to confirm this view experimentally. First, we will present certain data clearly showing that the cells of a pure culture of B.mycoides are far from equivalent in their temperature be- /280
havior.

If a suspension of bacterial cells is cultured on MPA, it will be found that only an insignificant fraction develops at temperatures close to the maximum. Table 90 gives the data of an experiment in which two cultures of B.mycoides, isolated from Moscow soils, were tested by this method. This also delineated, in some sense, the adaptability of bacteria to elevated temperatures. No doubt, the process involved is that of a "selection" of thermostable cells and of the death of millions of unadapted bacteria. Thus the concept of "plasticity" of the bacterial cell should be used with great caution.

TABLE 90

GERMINATION OF B.MYCOIDES CELLS AT VARIOUS TEMPERATURES

Culture No.	Medium	Count after 24 Hours at Temperature (in °C)			Count after 48 Hours at Temperature (in °C)		
		30°	36–37°	38°	30°	36–37°	38°
1	MPA	100	105.5	6.1	100	103.0	7.5
	MPB	++	++	+	++	++	+
2	MPA	100	0.3	0.15	100	2.3	0.16
	MPB	++	+	+	++	+	+

Note. 1. The number of cells grown on MPA at 30° is taken as 100.
2. The broth was inoculated with the same quantity of the culture as the Petri dishes.
3. ++ denotes good development on MPB; + satisfactory development on MPB.

This experiment also indicates that, for accurately defining the true maximum temperature of most of the bacterial cells of a given culture, a procedure different from the ordinary must be used. The existing technique demonstrates the ability of individual cells of a given culture up to a certain temperature limit, but does not characterize the majority of the bacterial cells. For example, the Moscow culture No.2 shows some growth on broth at 38° C, although the mass of the cells of this same culture stops growing at 36 - 37° C.

It follows from these observations that, occasionally, the thickness of an inoculation material may alter the maximum temperature of growth of the culture. In fact, the greater the number of bacterial cells with which the medium is /281 inoculated the greater will be the probability of encountering a heat-resistant cell in the inoculation material. We also confirmed this in the following experiment: A streak culture was made on an agar medium in Petri dishes by inoculating it with suspensions of B.mycoides of varying density. The dishes were placed for 24 hours in the incubator at different temperatures. The result of the bacterial count on the experimental medium is given in Table 91.

Many dishes inoculated with diluted suspensions of B.mycoides showed no growth at 37° C. When these same dishes were kept at 30° C, colonies appeared along all stab lines. Hence, it follows obviously that the dishes did contain cells of B.mycoides, but that these included no heat-resistant cells.

In the following experiment (Table 92), we attempted to establish the percentage of B.mycoides cells (Moscow culture No.2) germinating at 37° C. For this purpose, the Petri dishes were inoculated with varying numbers of B.mycoides /282 cells, after which the number of colonies grown at 30 and 37° C was determined. As indicated, the percentage of "heat-resistant" cells of B.mycoides is not high: At 37° C, not more than 3% of the cells in the Moscow culture of B.mycoides developed.

TABLE 91

EFFECT OF THICKNESS OF INOCULATION MATERIAL ON DETERMINATION OF THE MAXIMUM TEMPERATURE FOR B.MYCOIDES

Temperature	Culture No.	Inoculation of Petri Dishes with Suspensions Containing the Following Number of Cells per cc (in Thousands)				
		75 000	700	50	10	1
30°	1	+	+	+	+	+
	2	+	+	+	+	+
37°	1	+	+	+	±	—
	2	+	+	+	—	—

Symbols: + presence of growth.
- absence of growth.

When the microbes were artificially adapted to an elevated temperature, a series of successive transfers of the culture could be made at increasingly higher temperatures. This resulted in a selection of the thermoresistant forms. With such a method, Dallinger succeeded in considerably increasing the growth temperature of protozoa.

In our work on the temperature adaptation of bacteria, we attempted to adapt the Moscow culture of B.mycoides to a higher maximum temperature and thus to shift the position of the optimum temperature.

TABLE 92

DETERMINATION OF PERCENTAGE OF B.MYCOIDES CELLS GERMINATING AT 37° C

Estimated Factor	Number of Cells Placed on Petri Dish				
	7500	2500	500	200	50
Percentage of germinated cells of the culture	0.9	2.6	0.2	0.4	0.0

We were successful to an extent by gradually increasing the temperature of the incubator until it exceeded the maximum temperature of our race. Over a period of several months, with frequent transfer cultures, we were thus able to increase the maximum temperature of B.mycoides by only 2° C. We did not succeed in obtaining a more thermophilic form during the time of our experiment. How-

ever, it is well known that thermophilic forms of B.mycoides do exist in nature (Brotskaya).

Our experience has proved that it is not easy to obtain temperature adaptation of B.mycoides. This has also been noted by other authors (Imshenetskiy). Hence, it becomes obvious that the differences observed in the individual geographical races with respect to the temperature could have arisen only after a long time.

However, it is worth noticing that our culture grew at the same temperature at which the cells of the original form did not grow at all. This shows that the new environmental conditions (culturing at elevated temperatures) promoted the appearance, in the population, of cells with new properties.

These experiments on osmotic and temperature adaptation of bacteria permit the following conclusions:

1. The individual geographical races of B.mycoides do not behave identically toward an increased concentration of the medium. The southern races, with their higher intracellular pressure, grow better on saline media.

2. The great majority of bacterial cells survive only a minor increase in salt concentration of the medium. At stronger salt solutions, very few bac- /283
terial cells are able to grow which, nevertheless, creates a deceptive picture as to the readiness of adaptation of the bacterial culture as a whole to the conditions of the medium.

3. Adaptation of bacteria to an elevated concentration of the medium proceeds by selection of the deviant forms possessing specific traits that correspond to the properties of the medium.

4. Individual cells of a culture are able to grow at a temperature that stops the growth of most of the cells. The heat-resistant cells serve as a nucleus for the production of new temperature races.

When the culture temperature is gradually raised, mutant cells, with a temperature maximum appreciably higher than that of the original culture, appear among the bacteria.

CHAPTER X /284

CLIMATIC CONDITIONS AND TRANSFORMATION OF ORGANIC MATTER IN SOILS OF VARIOUS TYPES

1. Supply of Heat to Bacteria in Various Climatic Zones

As stated above, most soil bacteria are mesophilic. This group of bacteria is responsible for the conversion of organic matter in the soil.

In the previous Sections, we defined the temperature requirements of mesophilic saprophytic bacteria from soils of various climatic zones. It seemed attractive to us to analyze the supply of heat to such bacteria in their natural habitat. This, in turn, could yield interesting information on the conversion of organic matter in the soil.

To obtain a distinct idea as to the rate of microbiological activity in soils of various climatic zones, one must first compare the soil temperatures with the heat requirements of mesophilic bacteria. Disregarding a certain discrepancy between the temperature optimums for multiplication of bacteria and for enzymatic activity, we selected the interval of most rapid bacterial growth as their optimum temperature.

Table 93 is a comparison of the mean soil temperatures at various geographic localities with the approximate optimum temperatures of saprophytic bacteria.

Obviously, for almost all localities the optimum temperatures of bacteria are incomparably higher than the actual soil temperature. It is only at certain times, and even then only in the South, that these two temperatures might coincide, allowing the bacteria (if moisture is present) to manifest their potential energy completely. Under ordinary conditions, however, as demonstrated below, the microbiological processes in the soil cannot unfold their full potentialities because of the improper temperature, and their intensity diminishes increasingly on progressing from southern latitudes toward the North. /285

In fact, the soil temperature in the chernozem belt in summer stays on the average near 20°C, which allows the bacteria to flourish far more vigorously than at 10°C or less in the North. While the adaptive reaction of bacteria reduces the principal temperature points for soil bacteria in the North, it does not fully compensate those differences in the temperature of the individual soils observed on passage from South to North.

Considering the interval between soil temperature in the summer and optimum growth conditions for the bacteria in question, one must not focus attention on the absolute values alone. For example, a temperature drop by 5°C, from 30°C to 25°C, does not have the same marked effect on the energy of the microbiological process as a drop from 10°C to 5°C. Thus, the 17.6°C gap between these values in the soils of Kursk (34 - 16.4°C) can hardly be considered equivalent

to that observed in the soils of Arkhangel'sk, where this gap is 18.0°C. In the latter case, the rate of the bacterial process, as a result of a heat deficit, will decrease far more than in the former case.

TABLE 93

COMPARISON OF SOIL TEMPERATURES (IN °C) AND HEAT REQUIREMENTS OF SOIL BACTERIA

Locality	*Mean Soil Temperature in May-August*	*Optimum Temperature for Bacteria*	*Difference between 1st and 2nd Columns*
Arkhangel'sk	10.5	28.5	18.0
Leningrad	12.9	30.0	17.1
Moscow	12.7	30.0	17.3
Kursk	16.4	34.0	17.6
Sinel'nikovo	18.7	35.5	18.8
Armavir	22.4	35.5	13.1
Crimea	~ 24.0	37.0	13.0
Central Asia	. 30.0	38.0	8.0

In view of the fact that the various segments of the temperature scale are not of identical importance for bacterial processes, we attempted to apply the van't Hoff coefficient as a correction. This permitted an individualization of the value of certain temperatures in bacterial life and activity (cf. infra).

From the comparisons given in Table 93, however, we can state that, on /286 progressing from South to North, the gap between the optimum temperature of the bacteria and the ambient temperature increases. The adaptive activity of the bacteria thus does not completely cover the heat deficit under the conditions prevailing in the northern zone. Consequently, the microbiological processes should diminish in the direction from South to North. The distinct traces of depression of the microbiological processes in the soils of the northern zone are manifested primarily in the peculiarities of the organic matter there, where mobile components are relatively abundant (see Turin's monograph "Organic Matter in the Soil").

This depression is also reflected in the composition of the bacterial population of the soil. Thus, as already noted, the abundance of B.mycoides in the soil is completely determined by the rate of mineralization of the organic matter in it. This, in turn, involves the presence of bacteria of the B.cereus - B.mesentericus group in the soils, which latter group, according to all our data, replaces B.mycoides in the soils. The process of nitrification is regularly connected, over metabiotic relations, with the process of ammonification, and becomes vigorous only in soils where the latter process is sufficiently energetic.

Therefore, it is no accident that, in the tundra where bacterial processes are suppressed because of climatic conditions, there are few sporogenous bacteria in general and almost none of certain types (such as B.mycoides and B.mesentericus). However, nitrification does not take place here.

In the podzolic soils of the central belt, owing to a certain intensification of the processes, many spores of B.mycoides can be found. The relatively slight decomposition of organic matter also does not induce intense activity by the B.mesentericus group, so that it is rarely encountered here. Nevertheless, the general background permits functioning of nitrifying bacteria.

Toward the South, for obvious reasons, B.mycoides diminishes in the soils, but the group of B.cereus and B.mesentericus increases in number. Activity of the nitrifying bacteria is also greatly intensified.

These facts are merely the first guideposts illustrating the features of the conversion of organic matter under various climatic conditions. There is reason to expect that future work will permit a still more complete definition of the regular connection we have outlined between the composition of the soil microflora and the trend of the soil-forming process. /287

The reservation must be made that rapid mineralization, under otherwise favorable conditions, requires not only certain temperatures but also the presence of moisture.

It is thus entirely logical that no rapid decomposition of organic matter takes place in the soil of a warm climate, if this soil dries out exactly during the most favorable period for bacterial function so that the vital activity is retarded or stopped. The presence of excessive moisture may likewise lead to preservative processes.

Such periods of deficient soil moisture play an important role in the accumulation of humic substances in the soil. This question will be discussed later.

Since temperature and moisture are essential indices defining the concept of climate, it is natural that many investigators, specifically Dokuchayev, the founder of genetic agronomy, attached great importance to these factors in the formation of soil types.

In the United States, Hilgard (1902) demonstrated the dependence of soil-forming processes on the climatic features. The same factor was also reflected in a number of papers on general geography (Richthofen, Walter, and others). Many authors attempted to establish a mathematical relation between climatic indices and specifics of the soil-forming processes.

Lang (1920) discovered a certain relationship between location of the principal type of soils and the hydrothermal factor (rain factor). This ratio was expressed by the formula N/t°, where N is the mean annual rainfall and t° the mean annual temperature, in $^{\circ}C$. For the cold regions, Lang suggested to replace the mean annual temperature by the cumulative temperature of the hot months, divided by 12.

Based on this formula, $N/t^{\circ} = 160$ corresponds to the boundary between podzol and chernozem and the ratio of 40 to the boundary between latesolic and desert soils.

Using the Lang formula for USSR soils, Neustruyev found the following mean values of the hydrothermal factor:

for the podzol zone >	70,
for the chernozem zone	40 - 50,
for the chestnut-soil zone	25 - 40,
for the serozem zone	20 - 30.

Meyer (1926) recommended a modification of the Lang formula by introducing a moisture-deficit index. In that case, the above formula would take the following form: /288

$$\frac{\text{total annual rainfall}}{\text{deficit of atmospheric humidity}}.$$

Voyeykov and Brounov also mentioned a certain connection between distribution of soil type and climate. According to their data, the boundary of the chernozem belt coincides with the axis of the trans-tropical maximum atmospheric pressure.

In exactly the same way, Kaminskiy noted the dependence of the distribution of soil types and vegetation on the relative atmospheric humidity (from determinations made at 1 P.M.). For the European part of the USSR, he distinguished seven climatic zones.

Positive results, indicating the existence of a correlation between the climatic features of geographical regions and specific features of the soil-forming processes, were likewise obtained in the extensive studies by Marbut (1928 - 1935), Robinson (1924), and Krause (1933).

Jenny (1929), Gedroyts, and Vigner also connected the properties of the soil colloids, produced during soil formation, with the features of the various climatic zones.

Volobuyev (1945) recently made a number of generalizations on the correlation of soil formation processes and climate.

Certain authors criticized these concepts. Thus, it was pointed out that the geomorphological conditions and the geological history of the region have a greater significance in soil formation than the climate.

Stremme (1934), for example, believed that the following factors have a direct effect on the process of soil formation: wildlife, topography, accumulation of water, underlying rock, and human activity. He considered climate and geological age to be indirect and subsidiary factors. In this connection, he emphasized the fact that soils of various types can be formed at a given climate.

Academician Prasolov, in one of his papers, noted the narrowness of this

interpretation, since even both topography and vegetation are functions of the solar energy, which determines the properties of the climate.

Of course, the above concepts as to the relation between distribution of soil types and climatic features contain an element of relativity.

Academician Prasolov mentioned that critics of the zonal theory correctly object to the idea that soil zones are belts which exactly encircle the /289
globe. It is illogical that some types of soils should be zonal and others azonal, meaning that the influence of external physical forces must not be separated from the other factors affecting the process of soil formation. The founders of the zonal theory are somewhat guilty of this crude concept. Naturally, a conglomerate of many factors influences the process of soil formation. This necessitates revision of old and simplified schemes. The universality of climatic action, however, permits the definite statement that the climate is one of the primary factors in soil formation.

From our point of view, a large number of soil features resulting from the action of the climatic factor are mediated by the activity of soil microorganisms. In this Chapter, we will specifically discuss several examples confirming this thought.

2. Conditions of Humus Formation

In this Section, we will consider the features of decomposition and synthesis of organic substances in soils of various types, as related to their temperature and moisture.

We will start with two possible modes of organic matter conversion in the soil. In the first, the residues of animals and plants entering the soil are decomposed into more or less simple mineral or organic compounds.

In the second case, blackish humic matter, giving the soil its characteristic dark color, is formed from these residues.

The basic nature of the process leading to the formation of dark humic matter is still unknown, despite the colossal amount of work devoted to it over a period of 150 years, by several generations of research workers.

In older work (Sprengel, and others), humus is regarded as an entity composed of various compounds derived from the decomposition of animal and plant residues.

Later, numerous authors attempted to synthesize humus and to determine the chemical nature of its components by detailed analyses. This work helped to establish that the composition of humus is not uniform, but that humic acid is the most specific of a large group of organic compounds (Schmuck, Waksman).

The majority of modern scientists no longer consider humic acid as an /290
individual chemical compound but as a group of different compounds.

Turin believes it more correct to speak of humic acids which have several physical and chemical properties in common.

The recent hypothesis by Waksman, namely, that the structure of humic acid (its "nucleus") is based on lignoproteinate has become quite popular. However, as pointed out by Turin, no conclusive proof of this proposition has ever been given.

Speaking of the raw material for the formation of humic substances, the attempts to prepare "artificial humus" must also be considered. Of course, identity of artificially prepared "humic" compounds with the natural formations can be disputed, but it is a fact that dark-colored compounds have been prepared by various treatments from widely differing compounds. For example, as far back as the time of Bertello, humic compounds were prepared by heating an alcoholic solution of caustic alkali with carbon tetrachloride in a closed vessel. Liebig noted that an alkaline solution of lactose and tannin gives humic compounds when left standing in air. A similar picture was observed by Hardy in experiments on chloroform and acetone.

When carbohydrates and proteins are heated with concentrated acids, a brown or black sediment is formed (Trusov, and others). Samuely believes that the dark-colored substances resulting from the heating of proteins are formed by a secondary reaction between the amino acids and carbon-containing compounds (of carbohydrate type).

Maillard advanced the similar view that humic acid was obtained by the interaction of amino acids with sugars (at elevated temperature).

A number of authors (Hoppe-Seyler, Reinitzer, Eller, and Trusov) noted that the oxidation of compounds with a benzene ring yields compounds very similar to the natural components of humus. Such results were obtained in experiments on the oxidation of phenol, quinone, and hydroquinone in alkaline solution.

Trusov considered that phenol-containing substances, on oxidation by specific enzymes, yield brown and dark-colored products with the properties of humic acid.

He also stated that the conversion of lignin into humus takes place by oxidation of any components containing a quinone or polyphenol group, which yield condensation products of the type of oxyquinone. /291

It is well known that lignin, when treated with alkali, is converted in air to a dark substance, similar in properties to humic acids.

Gartner has shown that when tryptophan is boiled with sugar in 22.9% HCl, up to 86% of its nitrogen is converted into a humic complex. Other amino acids (arginine, histidine) behave like tryptophan.

Some chemists believe humus to be formed by the polymerization of furfurol. The latter compound can be obtained on treatment of carbohydrates, especially pentosanes and uronic acid complexes, with hot solutions of acids.

It has also been stated that mineral acids, acting on hydrocarbons, yield hydroxymethylfurfurol which is condensed to form humus. A similar condensation is believed to occur for furfurol.

The possibility of conversion of sugar, paraffin, glycerol, oleic acid, balkhashite bitumen, and other compounds into humic substances by slow oxidation at elevated temperature has also been demonstrated experimentally.

Thus, it is evident that humic compounds can be formed not only out of lignin but also from widely differing organic compounds. This proposition is fully justified by the Le Chatelier principle, according to which substances change their chemical composition, under changing conditions, into forms that are more stable under the new thermodynamic conditions. An X-ray study of humic acid has shown it to have a hexagonal space lattice of the graphite type. The graphite structure, under soil conditions, is exceptionally stable and is not destroyed even at 1000°C. Thus, in the formation of humus, chemical substances are converted into more stable forms.

Lignin carries the nucleus of the graphite structure and, on humification, concentrates its rings. Other compounds take a more complex path of conversion in the formation of humus. Humification, on the whole, is not a process of simplification but of complication of the chemical nature of matter.

In the natural environment, as noted by Slezkin, Waksman, and others, many organic compounds of plant and animal residues decompose rapidly and thus cannot serve as raw material for the formation of humus. This is true of the mono- and disaccharides, starch, and other compounds. The conclusion is drawn that humus is formed mainly from substances relatively difficult to decompose. For this reason, highly mobile substances participate in the formation of humus /292 only after they have first been converted by microorganisms into substances some of which seemed difficult to decompose and then serve for humus formation after death of the microorganisms.

The more stable compounds, such as lignin, chitin, wax, and the various hemicelluloses, can serve directly as source for humus formation, without first undergoing bacterial conversion. Lignin, which is present in substantial amounts in the Vegetable Kingdom, is of particular importance. Somewhat modified and combined with proteins, it forms the base of humus, the lignoprotein nucleus. According to Waksman, the lignoprotein nucleus constitutes up to 60 - 80% of humus. It is for the most part identified with humic acid.

Various authors mentioned the resistance of certain hemicelluloses to bacterial action and considered them the principal raw material for humus, asserting that their properties resemble those of the polyuronides.

This view of Slezkin and Waksman may be generally correct but, in our opinion, disregards certain propositions. Primarily, soil is a heterogeneous medium, and at many points of the system there may be zones so unfavorable to bacterial activity that the process of mineralization is extremely slow. In this environment, organic compounds (partially modified by the bacterial ecto-enzymes of the soil) may react with each other before they are destroyed by the bacteria. In this way, "zones of preservation" of organic compounds are estab-

lished and humic substances, no longer easily accessible to bacterial action, are formed. In such a medium, even relatively labile organic compounds may evolve into humus.

As we see it, humus formation is an ectocellular chemical process taking place in a medium which is external to the bacteria. Bacteria naturally catalyze this process, since their enzymatic activity breaks down the organic residues and indirectly favors the secondary reaction, namely, that of their combination with the humic substances. We assume that the latter reaction is possible only in the case of a certain depression of the bacterial processes. Otherwise the organic compounds are completely mineralized. The energy of formation of humus should be inversely proportional to the rate of bacterial processes in the soil, i.e., the formation of humus can proceed only if there is a certain suppression of microbiological processes. The environment in this case (with respect to temperature and moisture) must not prevent chemical reaction.

Experiments by various authors, including the present author, confirm /293 this view.

The experiments by Sorokina, for instance, indicate that if organic matter (mycelia of fungi) is introduced into a medium in which microbial processes are taking place, no humic substances are formed. After a certain depression in microbiological activity, the same raw material will yield an appreciable amount of humic substances. In a liquid nutrient medium, or in sand, the fungal mycelium is decomposed at 30° C without residue. Conversely, in poorly aerated kaolin, humic acid is formed. Strictly speaking, we have a similar phenomenon in nature. This was noted by Jenny, who derived a mathematical relationship between accumulation of humus in the soil and climatic factors.

Based on many years of study of forest soils, Feher concluded that, in the summer months when the soil temperature rises and the number of bacteria in the soil increases, the supply of humus in the soil layer decreases appreciably. The opposite picture is observed in the colder time of the year.

Löhnis and Green, based on experimental data, reached the conclusion that semi-anaerobic conditions, i.e., conditions which to some extent suppress bacterial activity, most favor humus formation. Chardet found that, whereas cellulose in the presence of air is oxidized to CO_2 and H_2O, it yields dark-colored products by condensation under anaerobic conditions.

Our view was fully confirmed by several experiments in which dark-colored humus-like substances were formed by autolysis of fungal tissues (Rippel) and grain (Mishustin). In these experiments, the decomposition of the tissue components was induced by the enzymes of the organism itself. The decomposition products were accordingly modified and, on reaction with each other in the absence of microorganisms, yielded humic substances at a relatively low temperature.

Even if it is assumed that, under the specific soil conditions, the carbohydrates, as readily mineralized compounds, cannot be converted into humus-like compounds, all of the above data would still leave open the possibility (at least in principle) of a direct conversion of relatively mobile organic compounds

into humus.

There is reason to believe that the chemical composition of humus will also vary with the rate of microbiological activity in the soil. It is not by chance that some authors note the extreme abundance of difficultly hydrolyzed compounds in the organic matter of southern soils, while the northern soils contain a /294 higher percentage of highly mobile compounds.

As shown by Motkin, the humus of the plowed horizon is considerably richer in lignin and poorer in protein than the horizons A_2 and B. From our point of view, this is easily explained by the fact that the processes of mineralization in the plowed layer are more energetic than in the horizons A_2 and B.

These considerations necessarily lead to the conclusion that readily mineralized substances have a definite part in the formation of humus. Obviously, depending on the environmental conditions in which the humus is formed, the relative share of these compounds in its formation will vary.

Suzuki's thesis that proteins, starch, and sugar can yield humus compounds should be mentioned in support of this view. Detmer, Czapek, and Bergius viewed cellulose as a raw material for the formation of humus in the soil.

The experiments by Marcusson clearly indicate that the difficultly decomposed lignin is not the only compound that enters into the composition of soil

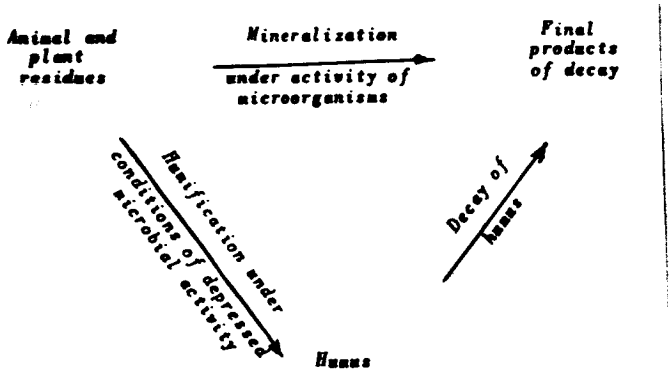

Fig.86 Principal Trends in the Conversion of Organic Matter in the Soil

humus. On determining the changes in decomposed wood, he found that, after a certain time, the percentage of lignoid compounds increased from 10.6 to 65, while the number of methoxyl groups increased only from 3.9 to 7.8%. This definitely supports the view that other substances besides the difficultly decomposed lignin enter into the composition of humus.

Finally, we must also mention the calculations by Sauerlandt who showed that the lignin entering the soil could not by itself make up the natural losses of humus. Experiments made by Kononova in the past three years likewise speak

for the major role played by highly mobile substances in the formation of humus.

Thus, we consider that humic substances may be formed from widely differing compounds in soils of depressed bacterial activity. Of course, this does not exclude their first being assimilated by bacteria, followed by secondary synthesis of humus after their death.

These considerations yield a scheme of the conversion of organic substances in the soil (see p.255).

The organic compounds in the soils of any climatic zone are converted by the two above routes. However, depending on the specific conditions, one or the other process may predominate. For example, in the tropics or subtropics, with their abundant heat and moisture, not only do organic residues mineralize rapidly but the humus formed in the "zones of preservation" is also subject /295 to decomposition. The final result is that the soils of this zone are not substantially enriched in humus.

In the soils of the chernozem belt, the spring periods of extremely rapid growth of vegetation give way to periods of insufficient moisture. In such dessicated soil, the activity of the microorganisms is of necessity greatly retarded. The individual groups of soil microflora have different moisture requirements. Here, the following series can be established, in decreasing order of moisture requirements: bacteria, mycobacteria, actinomycetes, fungi.

In our laboratory, Yenikeyeva found that bacterial growth at maximum hygroscopicity of the soil requires not less than twice the reserve of moisture. It follows that the bacteria in the soil assimilate about the same forms of moisture as the higher plants. Fungi can develop in a drier soil. Ferments are still active in soils, even at the minimum moisture levels that exclude the growth of microorganisms.

A quite similar phenomenon is also observed in the fermentation of tobacco and tea, when the soured plant tissues show extremely rapid fermentative processes, even in the absence of microbial life activity. It is therefore only natural to expect that the processes of humus formation will be intensified in southern soils, owing to the depression in the activity of microorganisms during the moisture deficit in the hottest period of the year (with the corresponding enzymatic catalysis).

In the more northerly belt (zone of podzol soils), the activity of the microorganisms is limited by the insufficiency of heat. The decomposition of the organic mass is not so rapid, so that secondary reactions, leading to formation of limited amounts of humic substances, are possible, although such /296 substances differ in nature from the humus of the steppe soils.

This is the general scheme which, in nature, is complicated by many factors of soil formation.

Below, we give certain calculations that clearly show the results of the hydrothermal conditions in the zones of soils of various types. We will not attempt here to establish indices indicating the distribution of soil types, and

merely wish to analyze the influence of the basic climatic factors of one aspect of the process of soil formation, namely, the fate of organic matter of the soil.

3. Hydrothermal Conditions of the Climate and Course of the Microbiological Process in Soils

On analyzing the hydrothermal conditions leading to the formation of various soil types, it would be more correct to start from data characterizing the soil temperature and the soil moisture during the hot time of the year. However, such materials are unavailable in practice. The soil temperature is measured in an extremely small number of places and by widely varying techniques (in some localities, the naked soil is examined and in other localities, the soil covered by vegetation). Exactly in the same way, it is difficult to collect data on the moisture of the soil (especially of virgin soil) during the vegetation period.

We shall therefore use the mean monthly atmospheric temperatures as criteria of the temperature conditions. These temperatures correspond roughly to the mean monthly soil temperatures.

We used the data on rainfall and evaporation to characterize the humidity of a given climate. By comparing these two indices, it is possible to establish the periods during which the soil is subject to excessive moisture or excessive drying.

Table 94 gives data, outlining the hydrothermal conditions in various localities in the European part of the USSR. Arkhangel'sk is taken as the characteristic point of the taiga-swamp region; Perm', Leningrad, and Moscow for the forest-swamp region; Khar'kov, Kiev, Dnepropetrovsk, Kherson, and Kuybyshev for the wooded steppe and the grassy steppe region; Astrakhan' for the desert-steppe region; and Batum for the subtropical zone.

The data presented yield a rather clear idea on the vital activity of microorganisms in soils of various types, assuming the following conditions:

1. It is assumed that the activity of microorganisms becomes appreciable /298 at a mean monthly temperature of 5°C.

2. When evaporation slightly exceeds rainfall, it is assumed that the soil does not dry out. The evaporation as read from Wild's evaporimeter is somewhat higher than that given by a large open basin. According to data by Poznyshev, the evaporimeter gives readings 1.24 times as high as the true values for the condition of the Yershovsk Experimental Station near Saratov during the summer period.

3. In the post-spring period with a deficit of moisture, the soil does not dry out immediately. We assume that for one and a half months after the onset of hot weather (above 5°C), in the absence of sufficient rainfall, the soil still retains enough moisture for the microbial activity. Drying of the soils

TABLE 94

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HYDROTHERMAL CONDITIONS FOR THE ZONES OF VARIOUS SOIL TYPES

Soil Type and Locality	Indices (In mm and °C)	Month of Year											
		Jan.	Feb.	March	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Soils of moist regions:													
Podzols:													
Arkhangel'sk	Rainfall	—	—	22	21	34	47	72	45	[illegible]	50	—	—
	Evaporation	—	—	13	20	36	50	60	45	30	[illegible]	—	—
	Temperature	—	—	—7	—1	6	13	16	14	8	[illegible]	—	—
Perm'	Rainfall	—	—	26	27	50	71	78	73	57	43	—	—
	Evaporation	—	—	21	42	76	70	72	57	42	23	—	—
	Temperature	—	—	—7	2	10	16	19	16	9	2	—	—
Leningrad	Rainfall	—	—	24	33	40	55	58	84	58	48	—	—
	Evaporation	—	—	10	24	44	63	63	46	31	18	—	—
	Temperature	—	—	—5	2	9	15	18	16	11	4	—	—
Moscow	Rainfall	—	—	27	35	52	62	78	71	55	54	—	—
	Evaporation	—	—	13	33	69	64	68	54	34	21	—	—
	Temperature	—	—	—5	4	12	17	19	17	11	4	—	—
Krasnozem:													
Batum	Rainfall	—	—	158	121	70	153	151	241	302	213	—	—
	Evaporation	—	—	35	80	120	120	130	160	100	60	—	—
	Temperature	—	6.1	9	12	17	20	23	24	20	17	12	9
Soils of trans. regions:													
Chernozems:													
Khar'kov	Rainfall	—	—	23	40	48	69	65	51	42	43	—	—
	Evaporation	—	—	15	28	76	69	82	68	51	28	—	—
	Temperature	—	—	—2	7	15	19	21	20	14	7	—	—
Kiev	Rainfall	—	—	44	47	49	76	76	54	47	50	—	—
	Evaporation	—	—	20	45	82	71	86	70	51	24	—	—
	Temperature	—	—	—1	7	14	18	20	19	14	8	—	—
Dnepropetrovsk	Rainfall	—	—	29	29	44	54	62	50	29	39	—	—
	Evaporation	—	—	18	61	108	95	114	121	89	47	—	—
	Temperature	—	—	0	8	15	20	22	20	15	9	—	—
Soil of dry regions:													
Dark-chestnut soils:													
Kherson	Rainfall	—	—	28	86	37	72	42	31	25	33	—	—
	Evaporation	—	—	30	65	90	80	102	104	78	38	—	—
	Temperature	—	—	2	10	17	21	24	23	17	11	4	—
Serozems and brown soils:													
Astrakhan'	Rainfall	—	—	9	16	15	19	12	10	16	10	—	—
	Evaporation	—	—	31	86	131	149	174	156	107	40	—	—
	Temperature	—	—	0	10	18	23	26	24	18	10	3	—

Note: Numbers in tabular data have been rounded off.

is faster in summer and takes 2 - 3 weeks.

These assumptions are based on the data collected by several experimental Institutes. Obviously, they should be further refined, separately for each climatic zone.

In the next period, lasting about one month, during which there is not sufficient rainfall, certain biochemical reactions may continue in the soil, but under strong depression of the growth of microorganisms.

Subsequently, if the drought continues, all microbiological and biochemical processes come to a complete standstill.

These periods, of course, are arbitrarily defined.

Thus, three periods can be distinguished in the life of the soil. Naturally, there are a series of transitions from one to the other. Their characterization is as follows:

I. Period of vigorous activity of microorganisms, which sets in at adequate moisture and suitable temperature. In this period, biochemical processes also continue in the soil.

II. Period of depression of microbiological processes, which sets in at a certain level of moisture deficit. This is characterized by predominance of chemical fermentation processes. The multiplication of microbes is considerably inhibited by lack of moisture*.

III. Period of complete suppression of soil processes, which sets in on strong desiccation of the soil.

The above assumptions permit a schematic profile of the hydrothermal conditions, characteristic for some of the principal soil types (Fig.87).

It will be found that, in the tundra zone where podzolic, gley-podzolic /299 and swamp soils are formed, there is no moisture deficit throughout the summer. We observe a similar picture for the forest-swamp region and its podzolic, gley, and swamp soils. The relatively low temperature, in addition, prevents the microorganisms from rapidly mineralizing the organic residues.

In this environment, secondary reactions leading to the formation and accumulation of a certain quantity of humus in the soil is entirely conceivable. In the presence of a descending flow of water, the disintegrated plant mass loses its basicity, and the products of decomposition assume an acid character. In the same zone of podzolic soils, small fractions are leached out of the upper horizon into the B-horizon. This leads to a relative enrichment of the upper horizons of the soil in silica and their pauperization in other oxides.

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* The weak growth of fungi and actinomycetes in relatively dry soil may be neglected.

In the chernozem zone, during the summer-autumn period, there is a pronounced moisture deficit. During the period of considerable soaking of the soil (spring and autumn), a relatively rich steppe vegetation develops. /300

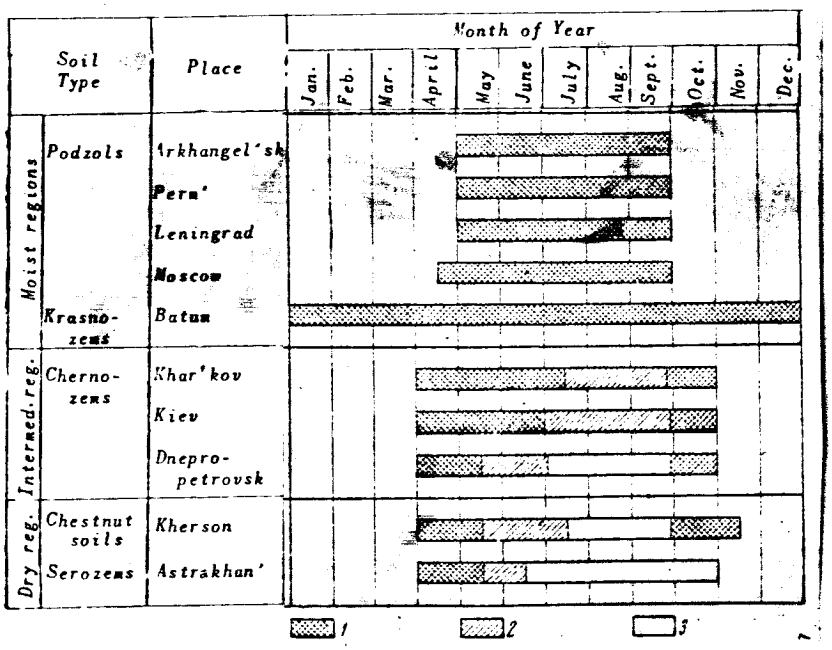

Fig.87 Course of Microbiological Processes in Various Soils (Schematic Diagram)
1 - Active microbiological processes continue. 2 - Microbiological processes are suppressed, but the ferments can still function. 3 - Both microbiological and enzymatic processes are suppressed.

Dokuchayev already noted the intimate relation between the distribution of chernozems, the climate, and virgin vegetation. He recognized the geographical distribution of chernozems as a consequence of climatic influence but not of the forest spread or the geological age of the country. Dokuchayev wrote: ... "Any vegetative soil, any chernozem has always been and will always be formed on any bedrock".

Vysotskiy likewise noted: "During summer and autumn the soils on the underlying rock are more or less extensively dried out to a considerable depth, primarily by the activity of the plant roots. In late autumn a new wetting of the soil begins, and the highest water content is observed in the middle or the second half of spring."

Academician Prasolov wrote: "Moderate moistening at constant alternation of the periods of soaking and drying, freezing and heating, swelling and contraction, development of vital processes and their extinction - all of this, which had been in effect for centuries and millenia, has given the development of the steppe soils a certain direction expressed in the characteristic structure or profile by which chernozems are readily distinguished from other soil

types."

The dominant influence of climate on the formation of chernozems explains the fact of their emplacement on various bedrocks, such as the granites of the Ukraine and southern Urals, the andesites of the Crimea and Transbaikalia, the basalts of the Trans-Caucasus, the sandstones, marls, limestones, etc.

Thus, the luxuriant vegetation of the springtime starts withering in the arid period of the year. Obviously, the phase of vigorous microbiological processes finds its end here. The moisture deficit will stop the vital activity of the microorganisms, while biochemical processes, under participation of microbial enzymes, may still continue for a relatively long time in chernozem soils. This period of slower multiplication of microorganisms - without excluding, however, the action of their endoenzymes and ectoenzymes - is in principle novel for the chernozem soil and explains the considerable volume of the humification processes. The dominance of the biochemical processes over the destructive processes of organic matter, in the hotter season, leads to their humification. At the same time, in the spring-autumn wet and hot periods, the most labile part of the humus is mineralized, thus increasing its ligno-humic fraction.

At some sites within the chernozem belt, as indicated in Fig.86, not /301
only biological but also biochemical processes may die down in the summer months.

The lack of moisture explains the slight change in the silicate portion of the chernozem soil by eluvial processes. Its relatively high content of cations (mainly calcium) favors the coagulation of the humic compounds as well as the formation of aggregates of closely linked organic and inorganic matter.

The chestnut-soil zone has a severe continental climate. Here extremely hot and dry periods give way to relatively wet ones. The thickness of the A- and B-horizons of dark chestnut soils correspond to the lower boundary of the winter-spring soaking, to the limits of twice the maximum hygroscopicity.

The presence of certain moisture reserves permits the development of an associated vegetation on chestnut soils. The period of their drying starts later. In the first phase, this is connected with depression of the processes and then with a new stoppage of microbial and biochemical processes.

Thus, the hydrothermal conditions in the zone of chestnut soils resemble those in the chernozem belt with a more pronounced moisture deficit. The humus of chestnut soils is therefore very similar in chemical composition to the humus of chernozems, and for entirely obvious reasons the former soils are distinguished by a shorter profile. The adequate calcium content in the soil favors the coagulation of humus and the formation of the soil structure. Eluvial processes take place in chestnut soils with a pronounced moisture deficit.

There is still less rainfall in the desert-steppe region. Here, after a brief spurt in spring, the vegetation begins to experience a depression of microbiological processes and a long period of drought. The relatively minor increase in plant mass is responsible for the slight accumulation of humic substances in the soils of this zone. The negligible rainfall and the high evaporation

from the soil result in the formation of solonchaks in many localities.

Local conditions (groundwater level, faults, etc.) may greatly affect the trend of the soil formation processes.

The exceptional amount of rainfall in the subtropical zone permits vigorous microbiological processes over long periods of time. No significant accumulation of organic matter, despite the extensive growth of the plant mass, can take place in such an environment, although the formation of humus is possible in /302 individual microzones of the soil, because of insufficient aeration and anaerobiosis. The active eluvial processes result in the formation of profiles of the type characteristic of krasnozems.

TABLE 95

RELATION OF NUMBER OF BACTERIA AND SUPPLY OF HUMUS IN BUROZEM SOIL OF SOUTHERN KIRGIZIA

Horizon	Irrigated Soils		Unirrigated Soils	
	% Humus according to Knopp	Number of Bacteria per Gram Organic Matter (in million)	% Humus according to Knopp	Number of Bacteria per Gram Organic Matter (in million)
A_1	5.6	150	15.2	9
A_2	2.8	45	8.8	10
A_3	2.5	13	6.4	3

On a single typical example, we would like to show that the intensification of bacterial processes in southern soils leads to an extensive depletion of the humus reserves. This again clearly shows the significance of the periods of insufficient moisture for the accumulation of humus. In one experiment, we studied the microflora of neighboring plots of burozem soil (southern Kirgizia). One of these was virgin soil, while the other was planted to an agricultural crop and was irrigated. We found that the irrigation, as expected, greatly increased the number of bacteria in the soil but, at the same time, caused a sharp drop in the humus reserves (Table 95).

The vigorous accumulation of humus is the result of the development of a luxuriant grassy vegetation in the spring period as well as of the depression of bacterial processes in the summer.

Below, we give an approximate calculation showing the amount of heat, in degree-days, received by various soil types during the following periods:

a) of vigorous bacterial processes;
b) of depressed microbial activity, at still possible enzymatic function;

c) cessation of microbial and biochemical activity.

The above assumptions were also used in these calculations.

The microbiological or biochemical activity, during some definite period, cannot be correctly determined without taking account of its increase with /303
rising temperature. For this reason, together with the calculations of the degree-days, we also present relative data characterizing the energy of the process, with allowance for the van't Hoff coefficient. In the latter calculation, we assume that each successive degree with rising temperature intensifies the bacterial or the biochemical process according to the van't Hoff law. Starting from this assumption, one must recognize that the temperature scale in the zone of high temperatures is more significant for activation of the biochemical and microbiological processes of the soil than in the zone of low temperatures. With increasing temperature, each degree has a greater intensifying effect on the processes taking place in the soil.

Thus, in applying the above correction for the temperature activation of the processes, their rate in the soils of various climatic zones will be accurately represented.

TABLE 96

HEAT SUPPLY OF VARIOUS SOILS

Zone	Heat in Degree-Days Received by			Mean Supply of Heat in One Hot and Wet Month (Degree-Days)	Heat Balance of Soils with Correction for the Van't-Hoff Law	
	Moist and Hot Period (Bacterial Processes Possible)	Hot Period with Moisture Deficit (Enzymatic Processes Possible)	Dry and Hot Period (Stoppage of both Bacterial and Ferment Activity)		Relative Quantity of Heat for the Hot and Wet Period	Part of Heat for One Hot and Wet Month
Podzols of northern belt	~ 2000	—	—	300—350	10	0.20 (1.0)
Podzols of central belt	. 2500	—	—	380—430	1.5	0.25 (1.25)
Chernozems	. 2000	~ 1000	~ 1000	420—450	1.5	0.35 (1.75)
Serozems	. 1000	. 1000	. 2000	420—450	0.8	0.35 (1.75)

In these calculations, we were guided by the general considerations noted at the beginning of this Chapter and by the fact that the biochemical activity of microbes strongly increases at an elevated temperature. This proposition has already been discussed above in the Chapter: "Energy of Multiplication among Geographical Races of B.Mycoides".

Table 96 gives the data obtained from these calculations. It will be noted

that the figures expressing degree-days are determined by the area plotted on the graph, the abscissa axis representing the time in days and the ordinate /304 axis the mean daytime temperature.

In calculations, connected with the application of corrections for the van't Hoff law, we made the corresponding change in plotting the ordinates. Here, each successive degree was spaced from the preceding position by the standard distance, increased by the van't Hoff factor. The area enclosed by the curve for the hot and wet period for the tundra was taken as unity. The corresponding periods for the other zones were compared with this standard.

It is obvious that these calculations are highly schematic; further work is required for additional refinement. Nevertheless, even the first rough sketch permits several interesting conclusions. Thus, from the material in Table 96 it follows that, on gradually progressing toward the South, the soil receives an ever-increasing amount of heat for each warm and wet period. This is distinctly depicted in calculating the relative quantities of heat, with allowance for the correction for the van't Hoff law. In the southern zones, the period of function of the microbes for the warm and moist seasons is more limited, but in these relatively contracted periods the thermal factor operates at great intensity.

As indicated by these data, in the chernozems a considerable amount of heat is received by the soil during the period when energetic bacterial processes cannot take place owing to the lack of moisture. In these periods, if there is no strong desiccation, according to our conception, the formation of humic substances increases.

The same situation is observed in the zone of serozem soils, but with a shorter period of growth for the vegetation, which limits the accumulation of organic matter in the soil.

Thus, the data discussed in this Section permit the following conclusions:

1. The optimum temperature of saprophytic soil bacteria is higher than the soil temperature during the hot period of the year. This statement refers to the mean temperatures and disregards the possible daytime overheating of the soil.

Bacterial growth temperatures somewhat higher than the soil temperature protect the bacteria from dying out during the summer overheating of the ground layer.

2. The adaptive reaction to the climate does not compensate the heat deficit in the northern soils for the bacterial population there. On transition from South to North the gap increases between the optimum temperatures of mesophilic bacteria and the soil temperature during the hot period of the year.

This factor explains a certain slowing of bacterial processes in the /305 soils of the northern belt of the earth. This is reflected both in the composition of organic matter in the soil and in the features of the group composition of its microflora.

3. Considering the process of humification, the author comes to the conclusion that during depression of the bacterial processes in the soils, not only the lignin but also relatively mobile organic matter can be converted into humus.

Humification is understood as an ectocellular process which proceeds spontaneously, on the basis of the Le Chatelier principle. The environmental ferments may promote this process.

4. The author emphasizes the importance of periodic summer drying of the soils of southern zones. These periods are regarded as the intervals during a considerable portion of which synthetic processes take place, leading to the formation of humus. In this book, data are given that corroborate this point of view.

5. Calculations given demonstrate that, during the moist and warm period, the bacterial processes in the soil of southern zones are in all probability more intense than in the North. This is explained not only by the better supply of heat to the southern soils but also by the intensification of microbial processes with increasing temperature, in accordance with van't Hoff's law.

In addition, the southern bacteria, adapted to the elevated temperature, have a greater biochemical impulse.

6. It has been shown from several specific examples that the hydrothermal conditions of the climate determine the composition of the soil microflora. Even today, this permits an analysis of the trend of the soil-forming process by the aid of so-called indicator organisms (such as B.mycoides, the B.mesentericus - B.cereus group, the nitrifying bacteria, etc.).

7. The above conclusions are based on preliminary calculations, which must be further refined.

SUMMARY /306

In this book, we considered the question of the influence of the climatic factor on the properties of soil bacteria, most of which are considered cosmopolites. The data compiled in this monograph were mostly obtained in a study of Bacillus mycoides, but the generality of the adaptive reaction to the climate in the majority of the soil microflora is shown from a number of examples.

The author, on the basis of rather extensive material, comes to the conclusion that eco-types exist among the bacteria and regularly succeed each other on progressing from North to South. Thus, the general law established by Darwin and his successors for the higher organisms must also be extended to the world of microorganisms.

The traits of the eco-types are hereditary, and therefore we may speak of the existence of geographical and ecological races of soil bacteria.

The basic conclusions of this work may be formulated in greater detail in the following propositions:

1. For B.mycoides, the previously described forms have been termed rugose and smooth. These forms can each be considered as a group of closely similar variants.

We were able to show in the present study that MPA, which is generally used for colony typing, is not suitable for this purpose. We recommended a new nutrient medium containing 0.1% peptone and 0.1% K_2HPO_4. On this poorer medium, the structure of the bacterial strands stands out in greater contrast.

A study of a large collection of cultures of B.mycoides on the new medium has permitted a classification of the great variety of forms of this bacterium into the following main groups:

1. Rugose Forms:

   a) with random orientation of the strands;
   b) with annular structure of the strands;
   c) with strictly oriented strands.

2. Transitional Forms: /307

   a) with elliptically curved strands;
   b) with both elliptically curved and radial strands.

3. Smooth Forms:

   a) with diffuse strands;
   b) with anthracoidal strands.

On other media, these types of B.mycoides maintained their characteristic colony structure, but these features are more difficult to differentiate.

On potato agar, the appearance of the colonies of all variants of B.mycoides is almost identical to the naked eye, but microscopic examination shows the differing features of the colonial structure of the various subspecies.

2. A study of a considerable number of soil specimens showed that, in the northern zone (in the podzols), the usual rugose variants of B.mycoides are most often encountered. In the southern chernozems and chernozem-like soils, variants forming colonies with elliptically curved strands are predominant.

In southern soils (chernozems), a considerable number of the variant of B.mycoides with strictly oriented strands, never found in the North, are encountered. The usual rugose variants here exist in relatively small numbers. A similar picture is also observed on the burozems of the South.

In soils of the dry subtropics (serozems), diverse variants of B.mycoides are encountered, with frequent occurrence of the smooth forms.

It is highly interesting that the soils of the southern mountain chernozems contain variants identical with those met in the northern chernozems.

Thus, it may be stated that definite variants of B.mycoides are characteristic of definite soil zones.

3. In addition to the ordinary method of inoculation on solid nutrient media, the author recommends the method of placing small lumps of soil on the surface of agar media, to establish the abundance of B.mycoides in a given sample. In working with northern soils, the inoculation can be done on MPA. Serozem soils contain smooth variants of B.mycoides which often give diffuse colonies only on potato agar. Therefore, it is advisable to use potato agar in the analysis of such soils.

The analysis of an extensive series of soils of various climatic zones /308 has led to the conclusion that the individual soils of the Soviet Union differ greatly in their content of B.mycoides.

The presence of B.mycoides reflects the state of a certain fraction of highly mobile organic compounds of the soil. In soils where the process of mineralization is retarded (for instance in the taiga), there is almost no growth of B.mycoides.

In soils of the podzolic zone, where the prerequisites exist for the slow succession of the mineralization phases, the soil is greatly enriched in B.mycoides.

An excessively rapid succession of the decomposition phases of organic residues in the soil makes the existence of B.mycoides ephemeral.

This explains the relative pauperization of southern soils in B.mycoides.

4. It has been established that the cell diameters differ among the principal variants of B.mycoides. The thickest cells are exhibited by the smooth variants, followed by the intermediate forms, and finally by the rugose variants of B.mycoides which have the thinnest cells.

It should be noted that the cell diameter of B.mycoides does not remain constant throughout the ontogenesis. Studies on the width of the cell showed a considerable thickening on aging.

Swelling of the cell precedes the formation of a prosporangium and cannot be considered as the consequence of purely mechanical stretching of the membrane by the forming spore.

On rich nutrient media, all known variants of B.mycoides have the same cell diameter in youth. On aging, however, the cells of the smooth variants thicken most, followed by the intermediate forms, and, finally, the cell diameter of the rugose forms of B.mycoides changes least.

On poor nutrient media, even very young cells of the several variants of B.mycoides differ in width. On aging, nevertheless, the thickening of the cell which is the rule for the other media is also observed here. The cell of the rugose variants is thickened by about 15% of its original width, that of the smooth variants by 35%.

Thus, the cell diameter cannot be regarded as a constant trait which does not change throughout the cycle of bacterial development.

In the smooth variants, the cell is usually shorter and has rounded edges.

5. In this work we studied the relation to various carbon sources of a collection of strains of B.mycoides, isolated from very different soils of the USSR. Many of these cultures differed sharply in a number of properties. /309
Nevertheless, all of the variants (rugose, smooth, and transitional) were almost identical in their relation to carbon sources. Only among the rugose variants with strictly oriented strands did we find more individuals that did not at all or only slightly ferment saccharose than among the other variants.

This work permits the conclusion that the complex of fermentative properties in the natural variants of B.mycoides is less subject to the influence of the environment than the other properties of the bacterial cell.

6. Most of the B.mycoides isolated from the soils belong to the so-called sinistral forms. Only from the soils of mountain places (Armenia, Urals) were we able to isolate dextral forms of B.mycoides.

In this book, we also discussed the causes for the rotation of bacterial filaments in the B.mycoides colony. A number of indirect proofs indicates that, on the whole, this phenomenon is connected with the peculiarities of growth of the B.mycoides cell on the surface of a solid nutrient medium.

In the case of intercalary growth of the bacterial filament, the latter can curve only if one side of the component bacterial cells grows at a different

rate than the other side.

It is also obvious that the composition of the solid nutrient medium exerts a substantial influence on the curvature of the bacterial filaments in the B.mycoides colony.

Depending on the ingredients of the medium and the concentration of the agar, the bacterial strands in a B.mycoides colony either cease to curve and begin to grow radially, or form their mirror-image colonies. Thus, by proper selection of a medium of a definite composition, sinistral forms of B.mycoides can be transformed into dextral forms.

In the author's opinion, this phenomenon can be explained by the fact that the vectorial forces of the water film formed on the surface of the nutrient medium exert a certain influence on the curvature of the bacterial filaments. If the sense of these forces coincides with the rotation of the B.mycoides filaments, the colonies will have highly circinate forms. In the opposite case, there is either radial growth or formation of colonies of the mirror-image type. In some cultures of B.mycoides, apparently due to the strong curvature of the growing cells, reorientation of this kind is impossible. The optical configuration of chemical substances has no effect on the orienting forces of the water film.

The reorientation of colony structure under the influence of the medium composition is not hereditary.

7. A study of the position of the cardinal temperature points for the /310 geographical races of B.mycoides showed that, on progressing North to South, the positions of the optimum and maximum temperatures show a marked increase.

Corresponding summary data are given in the following Table:

Cultures of B.Mycoides	Position of Optimum Point, °C	Position of Maximum Point, °C
From northern podzols	26 - 28°	36 - 37°
From podzol zone	~ 30°	37 - 40°
From chernozem zone	32 - 35°	~ 42°
From serozem zone	~ 38°	~ 45°

The relation of a culture to the temperature is a firmly fixed trait, persistently transmitted to the progeny.

8. There is a relationship, expressed by the equation of a straight line, between the position of the optimum and maximum temperatures of the geographical variants of B.mycoides on the one hand, and the indices characterizing the temperature conditions of the climate (July and mean annual air temperature, cumulative temperature for the hot period of the year), on the other hand.

9. The statement in item 8 above, permitted the use of specific traits of individual races of B.mycoides for an analysis of the origin of various seed material. B.mycoides is always found on grain, so that it is easily isolated from this material.

10. We have found that the southern races of B.mycoides not only have an elevated optimum temperature but also multiply more vigorously at their own optimum temperature than the northern races at theirs. This might partly explain the more rapid mineralization in southern soils since, on the whole, the energy of bacterial processes is connected with the energy of multiplication of the bacteria.

At the same time, our experiments show that certain oxidative ferments (catalase) show more vigorous function in the cells of the northern races of B.mycoides.

11. Analyses of the temperature conditions for the development of various saprophytic bacteria, isolated from soils of the various climatic zones, /311 demonstrated their pronounced adaptivity to climatic conditions.

Thus, also in B.mycoides there exists a simple relation, expressed graphically by a straight line, between the mean annual air temperatures (and the cumulative temperature for the hot period of the year) and the optimum growth temperature of the bacteria.

12. In some bacteria, evidently belonging to the aquatic forms and not fully adapted to the soil conditions, no pronounced adaptivity to environmental temperature conditions is noted.

The nitrogen-fixing azotobacter is of this type.

13. The fact that the great majority of saprophytic soil bacteria adapt to the climatic conditions indicates that thermophilic bacteria do not replace the mesophilic microflora in the soils of the southern zone. Extensive experimental material shows that virgin soils, even in the South, are extremely poor in thermophiles.

The soil is enriched with thermophilic microorganisms only after its cultivation by man.

14. Based on an analysis of literature data and own experiments, the author comes to the conclusion that the plasmolytic method of determining the osmotic pressure of bacteria yields unreliable results. Moreover, the inability of many bacteria to exhibit plasmolysis in weakly hypertonic solutions is noted. This is due to the fact that the bacteria have a readily shed cell wall and that the separation of the protoplast from the cell membrane occurs only in strongly hypertonic solutions.

This monograph also gives experimental data, suggesting the possibility of using the volumetric method of osmotic pressure determination in work on certain bacteria.

15. Determination of the osmotic pressure in the cell of geographical races of B.mycoides showed that this quantity may vary extensively. In cultures isolated from moist northern localities, the osmotic pressure of the cell does not exceed 2 atm. In cultures from southern soils, it rises to 15 atm or more.

The indices characterizing the climatic conditions are connected by a simple relation, expressed by the equation of a straight line, with the osmotic pressure of the B.mycoides cell.

The osmotic pressure of the cell in other saprophytic bacteria of the soil varies in exactly the same way as in B.mycoides.

16. The conditions for appearance of adaptive traits in bacteria are /312 analyzed. It is shown that selection is of decisive importance in this process.

17. The living conditions of bacteria in the soil of various climatic zones are analyzed in connection with the hydrothermal environment. Corresponding calculations make it possible to understand the situation leading, under some conditions, to a rapid mineralization of organic matter and, under other conditions, to an intensification of the processes of humus formation or to the preservation of plant residues.

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